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An Autoradiographic Study on the Uptake of Radiosulphate in the Rat Embryo[1]

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WITH FOUR PLATES

INTRODUCTION

IN the last few years various groups of workers have studied extensively the fate of radiosulphate in the animal body. Accounts of the research performed have recently been given by Boström (1953) and Boström & Jorpes (1954). If a small amount of radiosulphate is given to an animal most of it is rapidly eliminated in the urine and faeces. In the adult rat approximately 95 per cent. is excreted in this way in 5 days (Dziewiatkowski, 1949). Some of the retained radiosulphate is present as inorganic sulphate, and is probably incorporated as such, in the mineral part of bone (Engfeldt *et al*. 1954*b*). Labelled sulphate can be recovered in large amounts from the sulpho-mucopolysaccharides, and autoradiographic studies have disclosed that radiosulphate is retained to the greatest extent by cells and tissues known—or supposed—to contain these substances. Recently it has also been demonstrated (Holmgård, 1955) that a certain amount of radiosulphate enters the sulpho-cerebrosides. On the other hand, little sulphate is utilized in the synthesis of taurine, methionine, and cystine (Tarver & Schmidt, 1939; Boström & Åqvist, 1952) or, consequently, keratin. The information available is still incomplete, however; for instance, little is known about the possible intervening steps between inorganic sulphate and sulpho-mucopolysaccharides or sulpho-lipids.

In connexion with the incorporation of labelled sulphate into the sulpho-mucopolysaccharides, the uptake by the embryo is of considerable interest. It has been shown that metachromatic substances—presumably sulpho-mucopoly-saccharides—occur in large quantities in embryonic tissues as well as in other rapidly growing tissues (Holmgren, 1940, 1949). A transfer of radiosulphate from the maternal rat to the foetus was first reported by Layton *et al*. (1950). Boström & Odeblad (1953) briefly reported the incorporation of radiosulphate

[1] A preliminary report on this subject appeared in *Experientia*, **10**, (1954), 67.

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by rabbit embryos as revealed by autoradiography. The uptake of S^{35} varied markedly in different tissues, being highest in those presumably containing sulpho-mucopolysaccharides. Applying chemical and to some extent autoradiographic methods as well, Dziewiatkowski (1953) demonstrated that the amount of radiosulphate retained by the embryo was directly related to its degree of development, increasing with its age. He also found that the radiosulphate fraction insoluble in trichloroacetic acid increased from 40 per cent. at 10 days to 90 per cent. at 20 days. Some tissues of the embryo showed higher activity than their maternal equivalents. Embryonal cartilage, for example, contained 30 times the amount in maternal cartilage 24 hours after the injection of radiosulphate. Lately Amprino (1955) has investigated the uptake of S^{35}-labelled sulphate in early chick embryos during cartilage and bone formation.

The aim of the present investigation was a systematic autoradiographic study on the incorporation of radiosulphate by the rat embryo and a comparison of the results with those obtained by histochemical staining reactions.

EXPERIMENTAL METHOD

Some forty female albino rats representing various stages of pregnancy were given an intraperitoneal injection of 3 mc./kg. of carrier-free radiosulphate (obtained from A.E.R.E., Harwell, England) in 0·9 per cent. saline with 0·02 per cent. sodium sulphate. The animals were killed 48 hours later and the embryos were withdrawn. Their weights and C.R.-lengths served as a control of their age as estimated from copulation dates. Some forty embryos 7–22 days old and five new-born from different litters were used for autoradiography. The new-born rats were born at the end of the 48 hours that were allowed to elapse between injection and killing, the radiosulphate having been injected into their mothers. Specimens were also taken from the mothers (and non-pregnant females) for comparison.

The majority of the preparations were fixed in absolute methanol and the remainder were fixed in 10 per cent. formaldehyde containing 4 per cent. basic lead acetate. After dehydration in ethanol and clearing in xylene they were embedded in paraffin. Serial sections were cut 8 μ thick, and were alternately placed on glass slides (for staining) and on methacrylate slides (for autoradiography).

Autoradiography was performed with Gevaert Dentus Rapid film. The methacrylate slides were passed through xylene and ethanol and dried. They were then placed in contact with the X-ray film between cardboard strips in iron screw-presses for 45 days. The films were developed in Kodak DK-20. The resolution was 15–20 μ. For orientation one-third of the slides were stained[1] with haematoxylin and eosin or van Gieson connective tissue stain. One-third of the sections were stained in a 0·1 per cent. solution of Toluidine Blue O, C.I. 925, in 30 per cent.

[1] Certified stains from the National Aniline Division, Allied Chemical & Dye Corp., New York, U.S.A., were used.

ethanol for 30 minutes. The remainder was stained with periodic acid-leucofuchsin (periodic acid-Schiff, PAS) in a modification of the original Hotchkiss's method (1948) previously elaborated (Friberg *et al.*, 1953). All solutions were made in 70 per cent. ethanol and the reducing rinse was omitted. The Schiff-reagent contained 0·1 per cent. Basic Fuchsin, C.I. 677, decolorized by SO_2 from a cylinder. The sulphite wash consisted of 70 per cent. ethanol saturated with SO_2. The slides were left for 30 minutes in the periodic acid-solution as well as in the leucofuchsin reagent.

RESULTS

No significant differences between embryos fixed in methanol and embryos fixed in formaldehyde-basic lead acetate could be observed with regard to content of radiosulphate or histochemical staining reactions. As judged from the autoradiographs the youngest embryos retained only small quantities of radiosulphate which were evenly distributed. There occurred a steady increase and differentiation in the uptake of radiosulphate towards birth. The ratios between the uptake by different organs within the same embryo varied with its age. Thus no general statement valid for all organs can be formulated. At 11 days there was a distinct differentiation of the uptake of radiosulphate—areas of mesenchyme containing more than the other tissues of the embryos. During the second half of the gestation period the highest uptake of radiosulphate occurred in areas of mesenchyme or its derivatives, e.g. cartilage, tendons, heart-valves. It also appeared that the autoradiographic picture of the internal organs was greatly influenced by their content of mesenchyme. Uptake of radiosulphate, however, was also found without relation to mesenchyme, for instance, in the mucous glands of the digestive tract and in some parts of the central nervous system.

Mesenchyme

The first areas of mesenchyme to show an uptake of radiosulphate higher than other tissues of the embryo were those around the notochord and the neural tube and in the heart-valves. In embryos 16–22 days old, cartilage, tendons, fasciae, connective tissue septa, dermis, and tooth papilla showed a high uptake of radiosulphate in addition to the structures already mentioned. As a rule, there was a close relationship between the amount of metachromatic ground substance and sulphate retention. The tissue mast-cells which showed high incorporation of radioisotope were not visible in the autoradiographs before 17–18 days of age. Their presence could only be safely established in full-term embryos when they occurred in great numbers (Plate 3, figs. K, L, M; Plate 4, fig. O). Their granules exhibited a more or less distinct metachromasia and sometimes a PAS reaction.

Where cartilage was formed, a progressive increase of radiosulphate incorporation was recorded which ran almost parallel to the occurrence of metachromatic ground substance, but because of the limited resolution it could not be excluded that radiosulphate was present also in the cells. The ground

substance of cartilage showed metachromasia and weak to moderate PAS reaction. The chondrocytes showed little metachromasia but often contained granules which stained heavily with PAS.

During enchondral bone formation the uptake of radiosulphate diminished as cartilage was replaced by bone. Calcified bone thus showed a lower incorporation of radiosulphate (Plate 4, figs. O, P), but displayed a stronger PAS reaction. Intramembranous ossification was preceded by an accumulation of great amounts of mesenchymal ground substance showing intense metachromasia and moderate PAS reactivity. Simultaneously there was a high uptake of radiosulphate (Plate 1, fig. B, brain capsule).

Cardiovascular system

The uptake of radiosulphate in the heart-muscle was low during embryonic development and distinct metachromasia could not be observed. The PAS reaction, however, was markedly positive already in embryos 10 days old. The mesenchymal components of the heart, i.e. annulus fibrosus and heart-valves, showed a high uptake of radiosulphate in embryos of 14 days and older (Plate 1, figs. A, B). These structures were metachromatic. In the wall of the aorta moderate to large amounts of radiosulphate were retained from about 15 days onwards (Plate 2, fig. D). The uptake of the main arteries exceeded that of the main veins. Blood-cells were not found to incorporate any significant amounts of radiosulphate.

Alimentary system

Increased uptake of radiosulphate occurred in connexion with the downgrowth of the lip furrow band in the surrounding mesenchyme, which showed metachromasia and PAS reactivity (Plate 1, fig. B). In the teeth of full-term embryos and new-born rats a high uptake of radiosulphate was noted at the pulpodentinal border and in the tooth papilla (Plate 3, fig. L). The predentine was weakly metachromatic whereas a stronger metachromasia could be seen in the tooth papilla. The PAS reaction occurred in a similar way. In the tongue a moderate uptake of radiosulphate was found in the superficial layers, especially in the basal parts (Plate 1, figs. A, B). It appeared to be housed mainly within the subepithelial mesenchyme. A moderate uptake was found in salivary glands of full-term embryos and new-born rats. In the oesophagus a distinct uptake of radiosulphate occurred from 15 days onwards. The uptake was marked at 18 days, but much less prominent in new-born rats (Plate 1, figs. A, B; Plate 2, fig. D). This uptake appeared to have occurred mainly within the mesenchymal parts of the wall, which showed metachromasia and a moderate PAS reaction.

The stomach was found to take up moderate amounts of radiosulphate at 17–19 days. The retention of radiosulphate within the walls of the intestine was found to vary during different stages of development. In embryos 18 days old the highest uptake of radiosulphate was found in the metachromatic subepithelial mesenchyme (Plate 1, fig. B; Plate 2, fig. G). A lower uptake was

found in the epithelium. At birth the uptake of the mesenchymal parts of the intestine was less prominent. The intestinal epithelium was at birth producing a mucous substance that was intensely metachromatic and strongly PAS reactive. Considerable amounts of radiosulphate were found in the epithelium and intestinal contents (Plate 2, fig. H). Mucus with the same staining properties and high uptake of radiosulphate was also found in the intestinal lumen of some embryos 10 days old. It was not possible, however, to demonstrate any such mucus or uptake of radiosulphate at 14–19 days.

The uptake of radiosulphate in the liver and the pancreas was low during the complete gestation period. In the bile ducts a moderate uptake was found in the later stages.

Respiratory system

A great uptake of radiosulphate was found in the cartilages of the nose (Plate 1, figs. A, B), the trachea and the bronchi (Plate 2, fig. D). Otherwise the uptake of radiosulphate was insignificant in the respiratory passages. In the lung, the uptake was low during embryonic development.

Urogenital system

In the metanephros, a considerable uptake of radiosulphate combined with metachromasia occurred in the mesenchymal stroma around the pelvis (Plate 2, fig. F). The uptake of radiosulphate in the blastemal areas was low. Towards the end of the gestation period the uptake of sulphate within the pelvic stroma was less striking. The differentiation of the bladder, genital papilla, and vagina was associated with an accumulation of metachromatic substances and an increased uptake of radiosulphate in the mesenchyme (Plate 2, fig. G). The uptake of radiosulphate was low in areas rich in differentiated muscle-cells.

Nervous system

The uptake of radiosulphate in the peripheral nervous system was low during all stages of development. The peripheral nerves and ganglia exhibited little metachromasia and reacted weakly to PAS. In the central nervous system the uptake of radiosulphate was also low as a rule, but a higher uptake occurred in some parts of the brain at certain stages of development. In the CNS of embryos about 10 days old the uptake of radiosulphate was low and evenly distributed. At 15 days, however, a differentiated uptake could be observed (Plate 1, fig. A). At 17–18 days, there was a fairly high uptake in the corpus striatum and certain layers of the cerebral cortex (Plate 1, fig. B). On the other hand, the ependymal cells surrounding the lateral ventricles did not retain much radiosulphate (Plate 2, fig. E). No significant difference between grey and white matter was observed in the cord. Those parts of the brain retaining more sulphate displayed a stronger PAS reaction than did parts with lower uptake. The metachromatic reaction was somewhat variable. The chorioid plexus reacted strongly to PAS and showed a fairly high uptake of the radioisotope.

Sensory organs

Uptake of radiosulphate in the eye was observed in embryos from 15 days. The uptake in the optic cup and the corpus vitreum was low. The peripheral but not the central part of the lens contained moderate amounts of radiosulphate. The cornea and sclera and the eyelids showed a moderate uptake of radiosulphate and metachromasia in embryos 17 days old (Plate 3, fig. I). In the inner ear maximal incorporation of radiosulphate occurred in the cartilaginous labyrinth. The uptake in the membranous labyrinth was low. A moderate uptake was seen in the membrana tectoria, the gelatinous mass of the cupula, and the otolithic membrane. These structures showed little or no metachromasia, whereas the PAS reaction was quite strong (Plate 3, fig. K).

Skeleto-muscular system

In the autoradiographs the picture is dominated by the intense uptake of the cartilaginous skeleton. A decrease in the amount of retained radiosulphate was registered in areas of calcification as cartilage was replaced by bone, the uptake of calcified bone being lower. The uptake of radiosulphate within cartilage was not uniform. In cartilage undergoing calcification, the highest concentration of radiosulphate was found at the level of the proliferating hypertrophic cells. The periosteum was metachromatic and retained moderate to large amounts of radiosulphate. The bone-marrow presented a low retention of radiosulphate. In the skeletal muscles the uptake of radiosulphate was low, except for mesenchymal septa, which often contained mast-cells. Tendons retained large amounts of radiosulphate (Plate 4, fig. P). The articular capsules showed a moderate sulphate fixation (Plate 4, fig. O).

Skin

In the embryos at 10–15 days, a moderate uptake of labelled sulphate was present in the skin. At full term a low uptake was found in the epidermis, but the corium contained a moderate amount. The tactile hair follicles showed a high uptake especially in the outer layers, whereas hair-shafts did not absorb any sulphate. The corium contained numerous mast-cells with a high incorporation of the isotope (Plate 3, fig. M).

Extra-embryonic structures

In the pregnant uterus at 10 days, a high uptake of radiosulphate was found in the decidua and especially its basal parts, whereas the myometrium showed a considerably lower uptake (Plate 1, fig. C; Plate 3, fig. N). The uptake in these areas was paralleled by the occurrence of the PAS and metachromatic staining reactions. Similar relationships were seen in the later stages of gestation. The occurrence of a very high uptake was found to correspond to the presence of cells with metachromatic granules in the decidua beneath the placental insertion. These cells are probably identical with the 'specific cells' of Asplund *et al.* (1940).

More cells, however, were seen in the sections than on the autoradiographs. The granules of these cells were found to be PAS positive. The cells were larger than average tissue mast-cells and lay close to the vessels. They were present in large numbers at 10 days, but seemed to decrease in number towards birth.

The placenta showed low uptake of S^{35} before 11 days, but moderate amounts were incorporated in the later stages of gestation. The uptake was evenly distributed and only the walls of larger vessels stood out clearly. In the umbilical cord a high uptake of radiosulphate was often found both in or around the walls of the vessels. The rest of the cord showed a diffuse, weak metachromasia and moderate uptake of radiosulphate.

DISCUSSION

The indirect mode of administration of the isotope to the embryos makes certain precautions necessary in the interpretation of the autoradiographs. The uptake of radiosulphate will depend on both extra- and intra-embryonal factors. We found a steady increase in the fixation of radiosulphate towards birth. This may be due in part to a facilitation of passage through the placental barrier and in part to increased sulphate-retaining activity of the embryonal tissues. It is not possible to state exactly when the main uptake of radiosulphate occurred, but only that it took place during the 48 hours of intra-uterine life following the injection. This interval is indeed long in relation to the rapidity of embryological development, and during it the individual embryo may have undergone considerable changes.

The amount of inorganic radiosulphate still present after the interval between the injection of the isotope and the withdrawal of the specimens can be considered as low. The autoradiographs thus mainly visualize the distribution of the incorporated radiosulphate. The incorporation can be expected to have occurred into the mineral part of bone (in older embryos, as inorganic sulphate), into the sulpho-mucopolysaccharides and perhaps the sulpho-lipids (as ester sulphate). There was a striking parallelism between the occurence of metachromasia and retention of radiosulphate. Metachromasia *in vitro* with toluidine blue may be caused by a fairly large number of substances, containing acid radicals (Walton & Ricketts, 1954, and others), such as sulphate groups, phosphate groups, and carboxyl groups. It appears that the sulpho-mucopolysaccharides and to a lesser extent the sulpho-lipids, hyaluronic acid, and nucleic acids can be expected to have occurred in such amounts as to have caused metachromasia in the sections. This points in favour of an incorporation of the sulphate into the sulpho-mucopolysaccharides. Certain discrepancies, however, occurred between the distribution of metachromasia and uptake of radiosulphate. Thus the membranes of the inner ear were found to incorporate radiosulphate and to exhibit a very strong PAS reaction but very little or no metachromasia. Sulphate uptake in the membranes of the inner ear of new-born rats has previously been reported by Bélanger (1953). Our staining reactions seem to be in accordance with those

reported by Wislocki & Ladman (1955). It must be remembered that metachromasia is influenced by a large number of factors, e.g. temperature and presence of other compounds such as basic protein (French & Benditt, 1953, and others). Thus the lack of metachromasia in this case does not exclude the presence of sulpho-mucopolysaccharides.

The retention of radioisotope was not directly related to the presence of substances giving the PAS reaction, although structures taking up radiosulphate were as a rule PAS positive. Mast-cells, for example, showed a high uptake of S^{35} but a weak PAS reaction, whereas muscle-tissue had a low uptake of radiosulphate but gave a strong colour with PAS in many cases. PAS is stated to stain any compound containing the 1,2-glycol group —CHOH—CHOH— (hydroxyl groups may be replaced by amino or alkylamino groups) or its oxidation product —CHOH—CO. It is probable, however, that a substantial colour is produced in the sections only by high molecular substances of polysaccharide nature (Hotchkiss, 1948). Thus glycogen, heparin-mono-sulphuric acid and hyaluronic acid will be stained. On the other hand, heparin-trisulphuric acid and chondroitin sulphuric acid cannot be expected to react (Jorpes *et al.*, 1948). The absence of any direct relationship between fixation of S^{35} and PAS reactivity is due to the fact that the PAS reaction is given by a wide range of compounds, many of which do not contain sulphates. Besides, certain sulpho-mucopolysaccharides do not react.

The resolution of the autoradiographs did not always permit the exact localization of the isotope, whether intra- or extra-cellular or in a special cell layer. In most cases an extra-cellular retention is probable, as the incorporation of radiosulphate was parallel to the amount of ground substance present. The uptake seen in connexion with, for example, mast-cells and 'specific cells' of the decidua, however, appears to be intra-cellular. Certain authors have found an incorporation of S^{35} into the nuclei of cells (Odeblad & Boström, 1953). Our material did not permit observations of that refinement.

As a rule the highest uptake occurred in connexion with the mesenchyme and its derivatives. This uptake most probably occurred into the sulpho-mucopolysaccharides. The formation of cartilage involved an accumulation of metachromatic material. These areas also incorporated great amounts of radiosulphate. From the histochemical findings it seems probable that a large portion of the metachromatic and radiosulphate-containing material would be identical with chondroitin-sulphuric acid.

Enchondral and intramembranous bone formation as well as the formation of dentine commenced with an accumulation of metachromatic, PAS reactive ground substance exhibiting a high uptake of radiosulphate. Thus these three calcification processes seem to take place in a similar way from a histochemical standpoint. Similar findings have recently been reported by Bélanger (1954) and Engfeldt *et al.* (1954 *a*, *b*).

The most conspicuous cells of the connective tissue are the mast-cells. Accord-

ing to Holmgren (1946–7) mast-cells are not present in 14-mm. rat embryos but have appeared at the 20-mm. stage. In our material they could be safely identified in the autoradiographs only in the oldest embryos, i.e. older than 17–18 days. At this time, they incorporated large amounts of radiosulphate, as do mast-cells of adult tissues (Jorpes *et al.*, 1953). Metachromatic ground substance, incorporating radiosulphate, is undoubtedly produced long before any typical mast-cells can be seen in autoradiographs or sections.

In some way related to the mast-cells are the 'specific cells' of the uterine wall. Asplund *et al.* (1940) stated that the metachromasia of these cells was less alcohol-resistant than that of the mast-cells. They assumed that the 'specific cells' contain sulpho-mucopolysaccharides which are not as highly esterified with sulphate as heparin. Similar cells have been described in the human decidua by Wislocki & Dempsey (1948). The metachromasia of these cells was, however, removed with ribonuclease. We found cells probably identical with the 'specific cells' which, besides being metachromatic, also gave a positive PAS reaction and showed a high incorporation of radiosulphate. These cells thus showed some of the more important characteristics of the mast-cells. Possibly they contain heparinoid substances and are merely variants of mast-cells.

In the digestive tract of the embryo, uptake of radiosulphate occurred in connexion with the development of the mesenchyme concomitant with the production of mucous substances presumably of epithelial origin.

In some parts of the central nervous system an increased uptake of radiosulphate was found at certain stages of development. The correlation with metachromasia, however, was somewhat inconsistent, but a better correlation was found to PAS reactivity. This incorporation of radiosulphate may be due to sulpho-mucopolysaccharides. The presence within the central nervous system of a ground substance containing mucopolysaccharides has recently been claimed by Hess (1953) and Bairati (1953). It is thus tempting to assume that some radiosulphate might have been incorporated into sulpho-mucopolysaccharides. On the other hand, an incorporation into sulpho-lipids is plausible and would possibly be associated with the process of myelination. It is, however, not known to what extent sulpho-lipids occur in embryonic nervous tissues. Besides, it is probable that the sulpho-lipids are removed by the present histological methods (xylene-alcohols). No certain conclusions can thus be drawn as to the nature of the sulphate-incorporating compounds of the embryonic brain.

Evidence in favour of the incorporation of radiosulphate into myelin has recently been given by Ringertz (1956).

SUMMARY

1. The uptake of radiosulphate in rat embryos 7–22 days old was investigated with autoradiography and compared with the occurrence of metachromasia after toluidine blue staining and a positive PAS reaction.

2. The youngest embryos retained rather small amounts of radiosulphate, which was evenly distributed. In the older embryos the amount of retained radiosulphate was considerably higher and regionally well differentiated.

3. Marked changes in the autoradiographic pattern of certain organs occurred with growth.

4. The uptake of radiosulphate was as a rule paralleled by metachromasia with toluidine blue. No direct relationship was found between fixation of radiosulphate and PAS reactivity of the tissues.

5. Radiosulphate was found to be incorporated into cells and tissues containing inorganic sulphate (mineral part of bony tissues) and sulpho-mucopolysaccharides (mesenchyme, mast-cells, &c.).

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EXPLANATION OF PLATES

The pieces of film exposed to the radioactive slides were used as negatives from which prints were taken. The photographs in the following plates are such prints, and in them, therefore, the *lighter* the area the more intense is the radioactivity represented.

PLATE 1

FIG. A. Autoradiograph of a sagittal section of a 15-day embryo. A high uptake of radiosulphate is seen in the following cartilaginous skeleton parts: base of skull, nasal cartilage, hyoid bone, and vertebral column. A high uptake is also seen in the heart-valves. A moderate incorporation is present in the meninges, base of tongue, skin, aorta, oesophagus, and intestines. The uptake in the liver and muscular tissue, including myocardium, is low and even. A low but somewhat uneven uptake is presented by the brain. Magnification ×10.

FIG. B. Autoradiograph of a sagittal section of an 18-day embryo. A high uptake of radiosulphate is seen in the cartilaginous skeleton, heart-valves, oesophagus, and brain capsule. A moderate incorporation is found in the aorta, intestinal submucosa, brain, skin, and fasciae, and a low uptake in the lung, myocardium, liver, and muscle-tissue. Magnification ×10.

FIG. C. Autoradiograph of pregnant uterus at 10 days showing high uptake in the decidua, and low uptake in the placenta and the embryo. Note numerous white dots in the decidua below the placental insertion, probably corresponding to the 'specific cells'. Cf. Plate 3, fig. N. Magnification ×10.

PLATE 2

FIG. D. Autoradiograph of a sagittal section of the thorax of an 18-day embryo. The heart is seen in the centre of the picture. The myocardium and the blood-filled cavity have not retained significant amounts of radiosulphate. A high uptake is seen in the skin, the aorta (upper centre and lower left), and the tracheal cartilages. Maximal uptake in the oesophagus (upper left) and the ribs. Lung (lower left), liver (lower right), and thymus (upper centre) contain little radiosulphate. Magnification ×25.

FIG. E. Autoradiograph of a horizontal section through the basal parts of the skull of a 17-day embryo. Maximal uptake is seen in the cartilaginous skeleton. A moderate uptake of radiosulphate is seen in the meninges. The brain has retained moderate to large amounts of radiosulphate. A marked spatial differentiation of the uptake is seen. The lateral ventricles are seen in the upper central part of the picture. Magnification ×25.

FIG. F. Autoradiograph of kidney of an 18-day embryo. The pelvis, to the left, and the ureter, to the right, have taken up large amounts of radiosulphate. The uptake in the metanephric blastema and the lobulated liver is low. Magnification ×25.

FIG. G. Autoradiograph of a sagittal section from the lower abdominal region of an 18-day embryo. Maximal uptake is seen in the vertebral column in the lower corners. A high uptake is found in the genital papilla and bladder. In the intestines the retention is low in the mucosa, but high in the mesenchymal layer; no radioisotope is contained within the lumen. Magnification ×25.

FIG. H. Autoradiograph of intestines from new-born rat. In the left coil mucus-producing cells with a high S^{35}-incorporation are seen. Radioactive mucus in the lumen of the right coil. Magnification ×25.

PLATE 3

FIG. I. Autoradiograph of a horizontal section of the eye of a 17-day embryo. Maximal uptake in the cartilaginous skeleton. Moderate uptake in the skin (lower right), sclera, cornea, lens capsule, and mesenchymal tissue around the eye. Low uptake in the lens, corpus vitreum, retina, optic nerve, and eye-muscles. Magnification ×28.

FIG. K. Autoradiograph of inner ear of a 21-day embryo. Maximal uptake in the cartilaginous temporale anlage. A high uptake is seen in the mast-cells in the mesenchyme areas. A fairly high uptake is noted in the otolithic membrane and the gelatinous mass of the cupula. Low uptake in the membranous labyrinth. Magnification ×28.

FIG. L. Autoradiograph of a transverse section through a tooth from a 21-day embryo. A maximal uptake is seen in the cartilaginous upper jaw. A high uptake is seen in the dental pulp, at the pulpodentinal border, and in mast-cells of the mesenchyme (above). Magnification ×28.

FIG. M. Autoradiograph from the nose region of a 21-day embryo. Low uptake of radiosulphate in the epidermis and moderate uptake in the corium. Numerous mast-cells can be seen as white dots. The tactile hair follicles show a high uptake, especially in the outer layers, whereas the hair shafts have not taken up any radiosulphate. Magnification ×28.

FIG. N. Autoradiograph of pregnant uterus at 10 days. Very low uptake in the uterine lumen, placenta (lower end of section) and in scattered vascular spaces. Low uptake in the myometrium (upper end). A moderate to high uptake of the decidua and the decidual-myometrial border. In the centre of the picture adjacent to dark vascular spaces, white dots are seen—most of which probably correspond to the 'specific cells'. Magnification ×28.

PLATE 4

FIG. O. Autoradiograph of a section from the hind leg of a 21-day embryo. Note maximal uptake of radiosulphate in the epiphyseal cartilages. A fairly high uptake is seen in the calcified bone. Low uptake in the bone-marrow. The skeletal muscles show a low uptake. Numerous mast-

cells in the skin appear as white dots. The articular capsule and some vessels show a moderate uptake. Magnification $\times 28$.

FIG. P. Autoradiograph of a section from the hind leg of a new-born rat (22 days from conception). Maximal uptake is seen in the cartilage at the right end, whereas a considerably lower uptake is found in the calcified bone at the left end. The uptake in the muscular portion of the leg appears to be confined to mesenchymal septa. Such strands are seen to converge on a tendon, which shows a fairly high uptake. Magnification $\times 28$.

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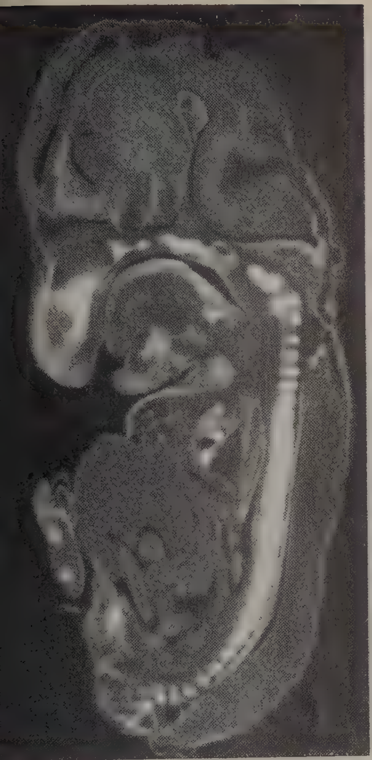

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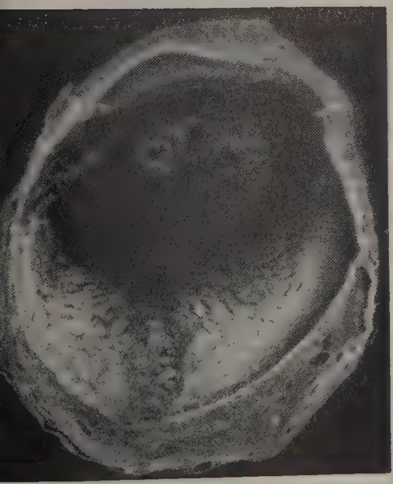

C

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Plate 1

D

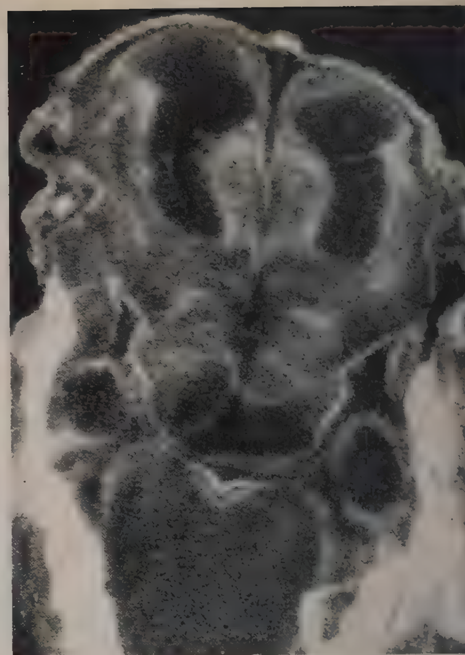

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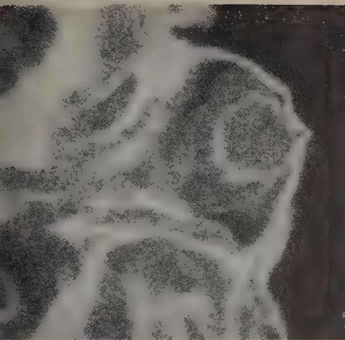
Plate 2



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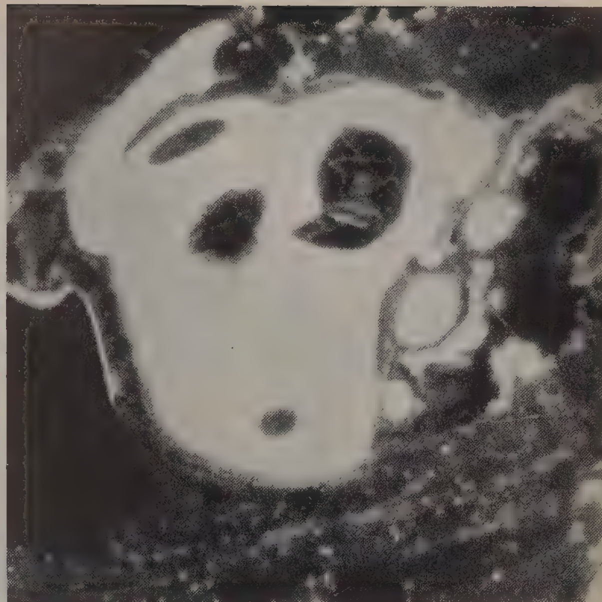

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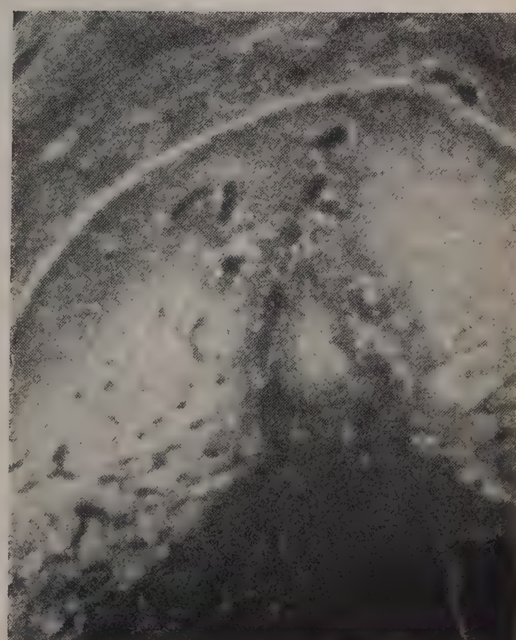

N

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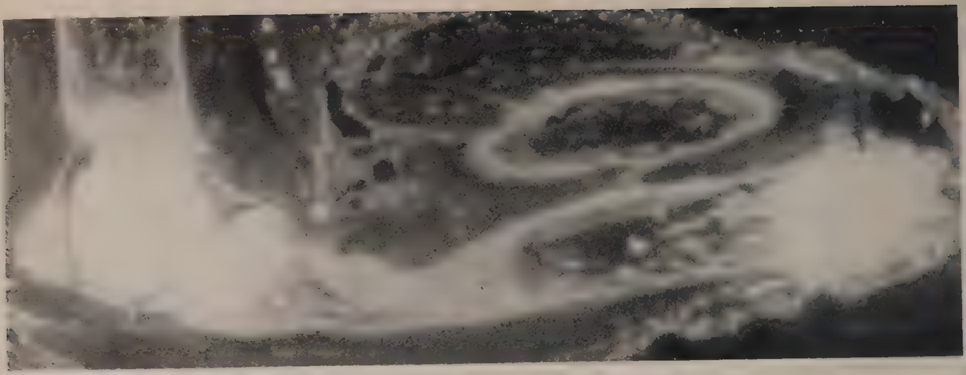
Plate 3



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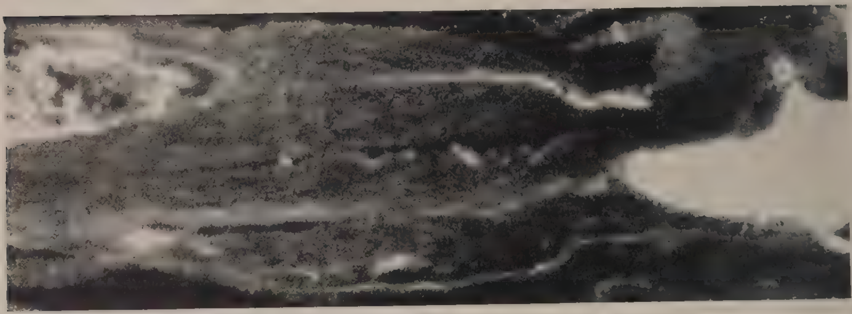

P

U. FRIBERG *and* N. R. RINGERTZ

Plate 4

Amino Acids in Developing Tissues of *Xenopus laevis*

by E. M. DEUCHAR[1]

From the Department of Anatomy and Embryology, University College London

WITH ONE PLATE

INTRODUCTION

INFORMATION about embryonic amino acids is of interest because of the possible light it throws on the differentiation of embryonic proteins. It is known from immunological work on amphibian and chick embryos (Cooper, 1946; Clayton, 1953; Ebert, 1950, 1952) that tissue-specific antigens are detectable before histological differentiation has taken place (Ten Cate & van Doorenmaalen, 1950). Although the chemical nature of antigenic differences is not yet properly known, recent evidence suggests that they may include differences in the number and arrangement of amino acids in N-terminal residues of the protein molecule (Porter & Sanger, 1948; McFadden & Smith, 1953; Putnam, 1953). For this reason, and on general grounds too, one may expect the formation of antigens in the embryo to be accompanied by altered arrangements of the protein-bound amino acids.

Radioactive tracer techniques have made it possible to detect the uptake of amino acids into embryonic proteins (Waddington & Sirlin, 1954; Feldman & Waddington, 1955), but so far this information has not been related to the appearance of individual antigens. In the amphibian embryo it has been shown that regions of rapid protein synthesis are also the most rapid absorbers of radioactive glycine and methionine from a saline culture medium (Friedberg & Eakin, 1949; Eakin, Kutsky, & Berg, 1951). This does not, however, necessarily imply that the single amino acids are taken up directly into proteins: they probably combine with other cell constituents first. More suggestive is the discovery by Gustafson & Hjelte (1951) that the concentrations of nearly all the free amino acids in sea-urchin embryos rise to a peak just before the gastrula stage, then decline during gastrulation just at the time when new antigens are forming (Perlmann & Gustafson, 1948). When so many free amino acids show the same trend in concentration, it seems likely that they are being used directly in the formation of new protein. If antigen synthesis is often accompanied by such

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striking amino-acid changes as this, it should be possible to distinguish different trends of amino-acid concentration in embryonic tissues as tissue-specific antigens appear.

Another reason for expecting developing tissues to show different trends of amino-acid concentration is that adult tissues have highly characteristic proportions of the different amino acids (Roberts & Frankel, 1949; Awapara *et al.*, 1950; Schurr *et al.*, 1950). Since each embryonic tissue has eventually to acquire the same chemical characteristics as the corresponding adult tissue, its amino-acid concentrations may during development gradually approach similarity to the adult.

It is possible by paper chromatography and microbiological assay to detect very small quantities of amino acid. Even so, a change in concentration of any one amino acid in a protein of high molecular weight may be on relatively such a small scale that it can pass unnoticed, especially when these microtechniques in which only small quantities of protein can be dealt with are used. So it is very difficult to establish with any certainty a series of changes in protein-bound amino acids throughout embryonic development. Those who have studied protein hydrolysates of *Rana pipiens* embryos (Holtfreter, Koszalka, & Miller, 1950; Kutsky, Eakin, Berg, & Kavanau, 1953) have not been able to show any significant changes during development. In *Xenopus laevis* embryos, the present author (unpublished) was also unable to detect any changes in protein-bound amino acids.

All the free amino acids of both adult and embryonic tissues appear to be present in comparably low concentrations, however, and it has been found possible to demonstrate changes in them by microtechniques. Eakin, Kutsky, & co-workers, for instance (1950 and 1953), observed fluctuations in the concentrations of individual amino acids in *Rana pipiens* embryos. Chen & Rickenbacher (1954) also detected free amino acid changes in embryos of the newts, *Triturus alpestris* and *T. palmatus*. But since all these authors confined their attention to whole embryos, it is not clear how their results may have been related to morphological differentiation. Any connexion between trends in free amino-acid concentration and the differentiation of embryonic tissues, can only show when different tissue primordia are analysed separately.

The following work provides some data on the distribution of free amino acids not only in whole embryos but also in parts of known developmental fate, and in larval tissues. Throughout, procedures have been kept as simple as possible, since the object was to obtain a sufficiently representative collection of data on various tissues for qualitative comparisons to be made between them, rather than to obtain accurate quantitative data.

In interpreting results of this kind it has always to be borne in mind that the 'pool' of free amino acid found in any tissue is in a continual state of flux. It is constantly added to by the diffusion or active transport of amino acid from other extra- or intra-cellular sources, and by the breakdown of protein. At the

same time, amino acids are lost from the pool by reverse processes of diffusion and active transport, and by incorporation into new protein. Changes in concentration of free amino acids are, therefore, connected with protein breakdown as well as with protein synthesis.

MATERIAL AND METHODS

Embryos of *Xenopus laevis* were obtained by injecting both male and female toads with the gonadotrophic hormone preparation 'Pregnyl' (Organon Ltd.). This species was found particularly suitable for the present work, in which abundant material was needed, since the adults react all the year round to hormone injections, and large numbers of eggs (500–1,500 per female) are laid at one ovulation. Moreover, the rapid development of the embryos at room temperature makes it possible to obtain samples of all stages from cleavage to hatching from one batch of eggs in a matter of 48–60 hours. Stage-to-stage comparisons could therefore always be made between samples from the same batch of eggs: an important precaution since different females laid different-sized eggs, and the same female could not be used for more than three successive injections without deterioration in her ability to lay.

Because of their rapid development, it was difficult to collect large samples of embryos that were all at exactly the same developmental stage. The stage-names used in the text, with corresponding 'Normentafel' stages (Nieuwkoop & Faber, 1956) are given below:

'Morula'	St. 5–6½	inclusive
'Blastula'	,, 7–9	,,
'Early Gastrula'	,, 10–10½	,,
'Late Gastrula'	,, 12–12½	,,
'Neurula'	,, 17–19	,,
'Early Tailbud'	,, 26–27	,,
'Hatched Larva'	,, 33–36	,,

Three series of samples were collected for analysis of free amino acid. Series 1 comprised whole embryos of morula to early tailbud stages. Series 2 comprised embryos ranging from blastula to hatched larva, which were dissected into different regions to be analysed separately. Series 3 consisted of parts of the month-old larva.

The embryos of series 1, that were to be analysed whole, were collected without removing their jelly or vitelline membranes. Besides speed, this method had the advantage that distilled water instead of saline could be used as collecting medium, since with their jelly on, the embryos survive in distilled water. Death and subsequent autolysis had to be avoided, while it was also desirable not to use saline as it caused streaking of paper chromatograms. It had been found by previous trials that the jelly itself contained no detectable free amino acid and would not therefore affect the results of the analyses.

Since the embryos of series 2 had to be demembranated in order to dissect them later, they were dealt with in groups of not more than 12, which were pipetted into a tube on an ice/salt bath at -10° C. before there was time for any autolysis to take place. (The ice/salt bath had to be used because it had been found that even at 0° C. some autolysis took place during the time needed to collect each sample of embryos.)

The larvae of series 2 and 3 were collected in distilled water.

After temporary storage at -15° C. each sample was frozen-dried. Specimens of series 2 and 3 were then dissected into the parts to be analysed separately. Dissection was difficult owing to the brittleness of the material, but the certainty that loss of amino acid could not occur either by autolysis or by diffusion made the freeze-drying method preferable to dissecting living specimens.

Before extracting the free amino acids each sample of the frozen-dried material was weighed. It was then pounded up in a mortar on an ice bath and transferred as a suspension in 2 c.c. 80 per cent. ethanol to a centrifuge tube. After 2 hours' extraction (occasional stirring) at 2° C. it was centrifuged for 5 minutes at 7,000 c/s. The supernatant was poured off into a solid watch-glass and the residue re-extracted for 2 hours in 80 per cent. ethanol three times. This procedure removed all detectable free amino acid from the sample. The combined extracts were evaporated in the solid watch-glass at 60° C., then stored in the refrigerator.

For chromatographic analysis each extract was redissolved by a standard procedure in the minimum volume of 80 per cent. ethanol and transferred, using a glass capillary tube, to a spot 1 cm. diameter at the corner of a 19 cm. square of Whatman No. 1 filter paper. The paper was run as a two-dimensional chromatogram, using as the first solvent 80 per cent. (w/v) aqueous phenol to which 3 c.c. of 30 per cent. aqueous ammonia had been added per 500 g. phenol. A trace of sodium cyanide was added to the bottom of the tank before each run to prevent discoloration of the phenol (Dent, 1948). As second solvent, 70 per cent. propanol was used. Control chromatograms of known quantities of amino acids were run at the same time as each experimental group to locate the positions of the acids and to serve as standards for the quantitative analysis.

The quantity of each amino acid on the chromatogram was estimated by a slight modification of the procedure used by Giri & co-workers (1952). This method, although not as accurate as others recently described (Troll & Cannan, 1953; Isherwood & Cruikshank, 1954; Levy, 1954), proved adequate for the present work.

The papers were sprayed with 0·5 per cent. ninhydrin dissolved in absolute ethanol (this solution gave maximal coloration and, provided it was freshly made up, a consistent blue tinge). The sprayed chromatograms were dried in cool air, then left for 20 minutes at 60° C. for the colour to develop. They were kept in the dark at room temperature for 20 hours before beginning the estimations. Each spot was then cut out and dropped into a tube containing 4 c.c. of Giri's copper sulphate-alcohol solution. The tube was shaken well, and the colour

allowed to elute from the paper for 1 hour. The quantity of colour present was estimated on a Hilger photoelectric absorptiometer, using an Ilford gelatin filter No. 4 (wavelength 540 mμ). Before estimating each experimental series, standard curves of absorptiometer readings were plotted from a control series.

Despite precautions to keep the procedure standard, the variability between experiments was such that the total free amino-acid values were nearly twice as high in some experiments as in others. This was probably mainly due to different sizes of embryos from different egg-batches and was not entirely corrected by expressing the data per unit weight, since the total nitrogen in large eggs tended to be proportionately lower than in small eggs. As it was impracticable to estimate total nitrogen in embryos from the same egg batches as were used for free amino-acid estimations, this variation could not be eliminated. There may also have been a variable error of up to 10 per cent. in the estimations of dry weight, as the frozen-dried material was somewhat hygroscopic and the atmospheric conditions during weighing were not always identical.

Amino acid values were, however, consistent within experiments, so that comparisons between tissues or stages within each experiment were consistent over the whole set of data. By subtracting the estimated amino-acid concentration in one sample from the concentration in the sample with which it was to be compared, a series of differences was obtained over the various experiments, and the mean of these differences was then tested for significance by a *t* test.

EXPERIMENTAL RESULTS

Series 1: Free amino acids in whole embryos

Groups of fifty embryos were collected in their jelly and extracted as already described. Each group of fifty provided the material for one chromatogram.

TABLE 1

Concentrations of amino acids in whole embryos; expressed in gamma/fifty embryos

	Morula	*Blastula*	*Early gastrula*	*Late gastrula*	*Neurula*	*Early tailbud*
Aspartic acid	9·48±2·00	5·18±1·00	2·69±1·00	3·57±0·61	3·62±1·34	2·34±1·33
Glutamic acid	10·97±1·13	9·67±1·75	7·97±1·13	5·39±0·78	3·94±0·65	3·46±0·54
Glycine	1·05±0·27	0·90±0·33	1·21±0·18	1·65±0·40	1·27±0·19	1·08±0·17
Glutamine	8·26±2·13	5·70±1·12	5·98±0·50	6·89±1·49	4·57±0·95	7·99±1·12
α-Alanine	1·69±0·36	1·20±0·21	2·04±0·38	2·98±0·93	2·42±0·96	1·09±0·19
β-Alanine	3·52±0·84	4·18±0·83	6·16±1·81	7·50±1·83	8·91±1·58	5·11±1·33
Valine	2·00±0·52	1·45±0·44	1·39±0·32	2·88±0·81	2·18±0·57	2·67±0·90
Leucine	3·03±0·64	2·59±0·67	2·35±0·83	6·43±2·12	4·99±1·28	5·55±1·86
Total	40·60±3·73	30·87±4·69	29·79±2·18	37·29±6·15	31·70±3·95	29·29±4·06

Each value is the mean, with standard error, of six experiments.

The following free amino acids were identified: aspartic acid, glutamic acid, glycine, glutamine, alpha-alanine, beta-alanine, arginine, valine, leucine, and

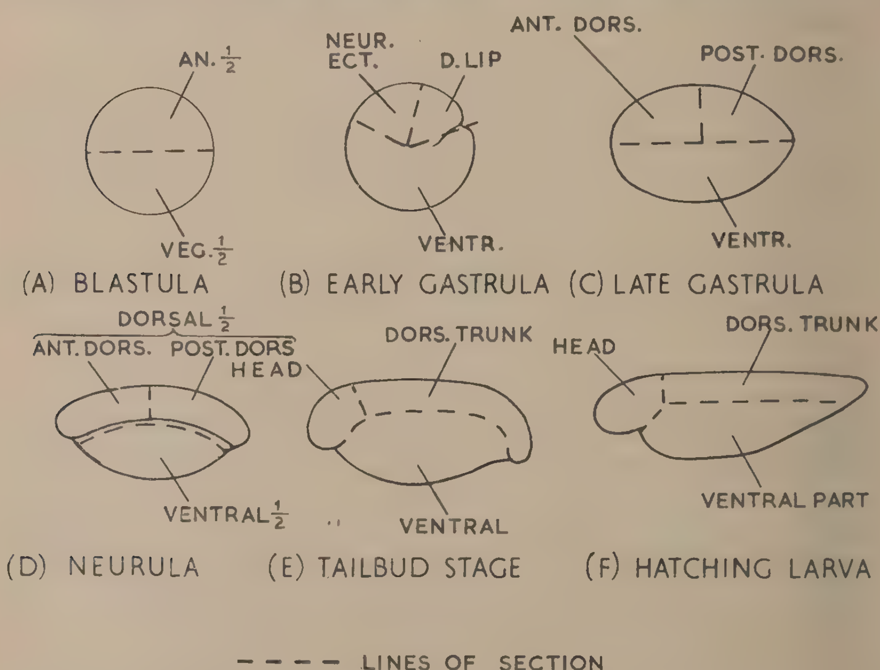
cystine. Lysine, tyrosine, and the tripeptide glutathione were sometimes also detectable. As can be seen from Plate, figs. A–F, there was apparently no qualitative change during embryonic development from morula to early tailbud stage, although some of the amino acids altered in quantity, as shown by the changes in size and intensity of the ninhydrin spots on the chromatograms.

The results of quantitative estimations on eight amino acids are given in Table 1 (means of six experiments). Comparing the total concentration of free amino acid between the developmental stages, it is found to be lower in the blastula than in the morula, and higher in the late gastrula than in the blastula.

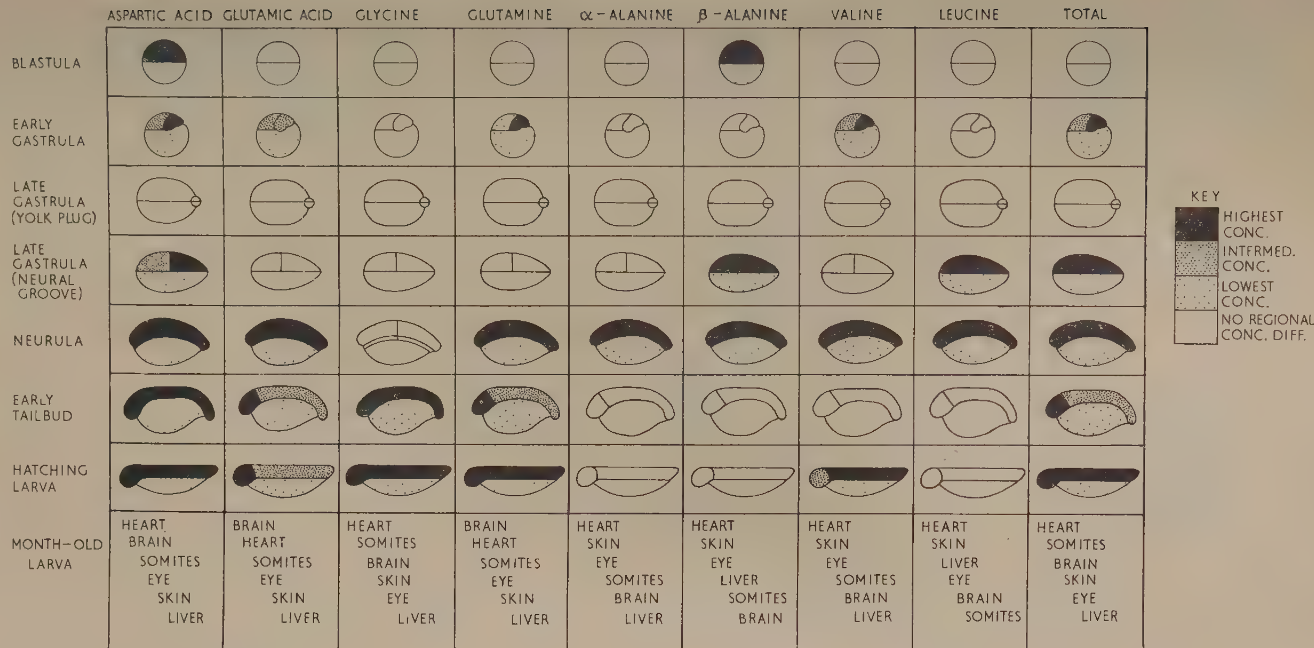
Both aspartic acid and glutamic acid, which are present in maximal concentration in the morula, decrease significantly between morula and early gastrula stages. The late gastrula differs from the early gastrula in having a significantly higher concentration of leucine, and the early tailbud stage differs from the neurula in having a significantly higher concentration of glutamine. (Other differences in intensity of spots on the particular chromatograms photographed in Plate, figs. A–F were not consistent throughout the whole experimental series.)

Series 2: Free amino acids in different regions of the embryo

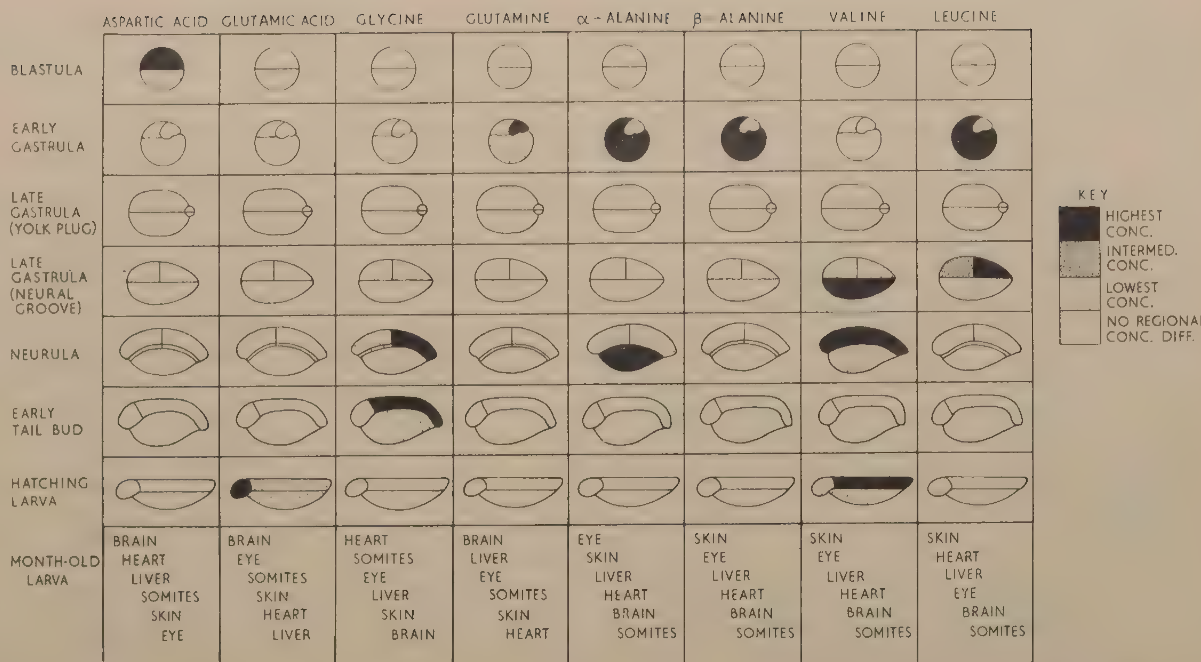
Because of the brittleness of the frozen-dried material the embryos could only be divided into two or three separate portions, as shown in Text-fig. 1. Only in



TEXT-FIG. 1. Diagrams of divisions of embryos into regions.



TEXT-FIG. 2. Significant differences in amino-acid concentration per milligramme of tissue between different embryonic regions are indicated by different types of shading. Where there were no significant regional differences, the diagrams are left white.



TEXT-FIG. 3. Significant difference in content of amino acid as a proportion of the total free amino acid between different embryonic regions are indicated by different types of shading. Where there were no significant regional differences, the diagrams are left white.

the early gastrula could this division be arranged to separate areas of precisely known developmental fate: at later stages each portion represented a complex of different tissues.

The regions which it was possible to separate did, however, show some interesting differences in their free amino-acid content. The results have been summarized in Text-figs. 2 and 3. In Text-fig. 2 the concentrations of amino acids per milligramme of tissue are compared between the embryonic regions. In Text-fig. 3 the amino-acid concentrations expressed as percentages of the total free amino acid are compared between the same regions, giving a better impression of which of the amino acids predominate, and also revealing some regional differences which do not appear in Text-fig. 2.

(*a*) *Blastula.* Comparing the concentrations of free amino acid per 10 mg., between animal and vegetative halves (Text-fig. 1A), it is found that the animal half contains more aspartic acid and beta-alanine than the vegetative half (Text-fig. 2). Expressed as percentages of the total free amino acid (Text-fig. 3) the concentration of aspartic acid is again higher in the animal half, but there is no significant difference between animal and vegetative halves for beta-alanine.

The total free amino acid per 10 mg. in the animal half was not significantly higher than in the vegetative half.

(*b*) *Early gastrula.* Embryos of this stage were divided into three parts: dorsal lip, presumptive neural plate, and ventral tissue (Text-fig. 1B).

Comparing the concentration of total free amino acid per 10 mg. between the three parts, it was found that the dorsal lip contained more than either presumptive neural plate or ventral tissue, and also that the presumptive neural plate contained more than the ventral tissue (Text-fig. 2). Of individual amino acids, both aspartic acid and valine were more highly concentrated in the dorsal lip than in either of the other parts, and were also more highly concentrated in the presumptive neural plate than in the ventral tissue. There was no significant difference in glutamic acid concentration between dorsal lip and neural plate, but each of these regions contained more glutamic acid than the ventral tissue. Glutamine showed no significant difference in concentration between dorsal lip and presumptive neural plate, nor between neural plate and ventral tissue, but there was significantly more glutamine in the dorsal lip than in the ventral tissue. Other amino acids showed no regional differences in concentration per 10 mg.

When the concentrations were expressed as percentages of the total free amino acid in each region (Text-fig. 3), the value for glutamine was again higher in the dorsal lip than in the ventral tissue. The percentage concentrations of alpha-alanine and beta-alanine were, on the other hand, lower in the dorsal lip than in other parts. The values for aspartic acid, valine and glutamic acid, unlike their concentrations per 10 mg., did not show any regional differences.

(*c*) *Late gastrula.* In a first series of analyses, carried out on embryos which still showed a small yolk plug and had not yet acquired a neural groove, dorsal and ventral halves (Text-fig. 1C) were compared for their free amino-acid content.

Neither the total free amino-acid content, nor the separate concentrations of individual acids, whether expressed per 10 mg. or as percentages of the total free amino acid, showed any significant differences between the two halves of the embryo (Text-figs. 2 and 3).

For the second series of analyses the dorsal half of the late gastrula was divided into anterior and posterior regions. This was easier to do accurately at a stage when the neural groove had appeared, because the full extent of the presumptive neural plate could then be seen by its paler pigmentation. The embryos used in this series of analyses were, therefore, slightly more advanced than in the first series. Values for amino-acid concentration in the complete dorsal half were calculated by pooling the data used for anterior and posterior portions. It is interesting that dorsoventral differences did appear, though they had not been detected in the slightly younger embryos of the first series.

The total free amino-acid concentration per 10 mg. in the dorsal half was higher than in the ventral half, and so were the individual concentrations of aspartic acid, beta-alanine, and leucine (Text-fig. 2). Aspartic acid was also more highly concentrated in the posterior part of the dorsal half than in the ventral half, but there was no significant difference in its concentration either between anterior and posterior parts of the dorsal half or between the antero-dorsal part and the ventral half. No other regional differences in amino-acid concentration per 10 mg. were detected.

The percentage concentrations (Text-fig. 3) of valine and leucine showed regional differences. There was a higher percentage of valine in the ventral half than in the dorsal half (though it was not different from the antero-dorsal part), while leucine showed a higher percentage concentration in the dorsal half than in the ventral half. The percentage of leucine was higher in the posterodorsal part than in either the ventral half or the anterodorsal part (Text-fig. 3).

(*d*) *Neurula.* As with the late gastrula, comparisons were made both between dorsal and ventral halves of the embryo and between anterior and posterior parts of the dorsal tissue (neural plate with underlying mesoderm) (Text-fig. 1D). In this section, however, the same set of data was used for both types of comparison.

As Text-figs. 2 and 3 show, there were very marked differences between dorsal and ventral halves of the neurula. The total free amino acid in the dorsal half was much higher than in the ventral half, and the separate concentrations of aspartic acid, glutamic acid, glutamine, alpha-alanine, valine, and leucine were also higher in the dorsal half. When these concentrations were expressed as percentages of the total free amino acid, however, only valine showed a higher value in the dorsal half, while alpha-alanine had a higher percentage concentration in the ventral than in the dorsal half, just as in the early gastrula.

Between anterior and posterior halves of the dorsal tissue, no significant differences in amino-acid concentration could be detected except for a higher percentage of glycine in the free amino acid of the posterior half.

(*e*) *Early tailbud stage.* The regional comparisons in this and in the hatched

stage were rather unsatisfactory: it was again only possible to divide them into dorsal and ventral halves or anterior and posterior parts of the dorsal-half (Text-fig. 1 E, F). These regions consist of a number of visibly different tissues, which were not distinguished in the analyses.

The dorsal tissues of the tailbud stage contained a higher total of free amino acid than the ventral tissues, and the individual concentrations of aspartic acid, glutamic acid, glycine, and glutamine were also higher in the dorsal tissue (Text-fig. 2). The percentage concentrations of these four acids did not, however, show any differences between dorsal and ventral halves (Text-fig. 3).

There were some amino-acid differences between anterior and posterior dorsal tissue. The total free amino-acid concentration of the head was higher than that of the trunk, as were also its concentrations of glutamic acid and glutamine, considered individually. A similarity to the neurula was that the percentage of glycine in the free amino acid of the trunk was higher than that of the head.

(*f*) *Hatched larva.* Comparing dorsal and ventral halves, it was found that the dorsal half contained a much higher total of free amino acid than the ventral half. Its concentrations per 10 mg. of aspartic acid, glutamic acid, glycine, and glutamine were also individually higher than in the ventral half, although these were not different between dorsal and ventral halves when expressed as percentages of the total free amino acid. In all these features the amino-acid distribution in the hatched larva resembled that of the early tailbud stage.

The head of the hatched larva contained a higher concentration of glutamic acid, though not of glutamine, than the trunk. Valine, on the other hand, was more highly concentrated in trunk than head. The percentage concentrations of both glutamic acid and valine showed the same antero-posterior differences as their concentrations per 10 mg.

Series 3: Free amino acids in tissues and organs of the month-old larva

The larvae were 4–5 weeks old and had been reared on a diet of liver juice and Bovril. They were fairly easy to dissect frozen-dried, as the dry, brittle structures could be peeled or broken away from one another more cleanly and quickly than when living.

The following parts were isolated and analysed separately for free amino acid: (1) central nervous system: this consisted chiefly of the brain, since the spinal cord in the *Xenopus* larva is very short, ending in a slender filum terminale; (2) eyes; (3) somite tissue of trunk and tail (with some of the tail skeleton and fin included); (4) heart, with truncus arteriosus, and the blood contained in it; (5) liver, still containing some unabsorbed yolk; (6) ventral and lateral skin; (7) gut, with contents.

As some larval organs (e.g. the heart) were very small, at least fifty larvae were needed for each set of analyses.

In Text-figs. 2 and 3 some visual impression of the results is given by arrang-

ing the names of the larval parts in descending order of their amino acid content. The mean values of all the data are given in Tables 2 and 3.

TABLE 2

Amino-acid concentrations in larval tissues; expressed in gamma 10 mg. frozen-dried material

Tissue	*Aspartic acid*	*Glutamic acid*	*Glycine*	*Glutamine*	
CNS	10·13±1·96	14·69±3·65	1·67±0·61	5·59±0·51	
Eyes	5·87±1·60	11·92±2·24	1·40±0·35	4·60±0·91	
Somites	7·66±1·40	12·64±0·94	2·26±0·48	4·85±1·20	
Heart	12·64±2·05	13·73±2·88	2·73±0·52	5·50±3·66	
Skin	5·95±1·34	7·58±1·11	1·96±0·89	3·67±1·35	
Liver	4·95±0·82	5·21±1·40	1·04±0·26	4·02±1·26	
Gut	7·79±0·98	11·39±3·60	2·29±0·85	7·72±2·43	
	α-Alanine	*β-Alanine*	*Valine*	*Leucine*	*Total*
CNS	2·27±1·03	9·80±3·52	2·22±0·88	3·16±1·00	49·55± 8·83
Eyes	3·71±1·46	12·27±4·30	3·50±0·98	3·55±0·85	46·82± 9·34
Somites	2·53±0·67	12·41±3·21	2·42±0·44	2·89±0·49	47·67± 2·95
Heart	5·18±2·86	16·24±6·06	5·40±2·62	15·49±4·19	76·91±20·32
Skin	5·16±2·16	12·92±6·11	4·74±1·52	9·43±2·15	51·42±10·80
Liver	2·16±0·67	11·11±3·45	2·13±0·64	3·81±1·10	34·42± 4·20
Gut	4·21±2·02	12·96±3·36	3·55±1·16	3·60±1·56	53·72±14·98

Values given are means, with standard errors, of 5 experiments.

TABLE 3

Amino-acid concentrations in larval tissues; expressed as percentages of the total free amino acid in each tissue

Tissue	*Aspartic acid*	*Glutamic acid*	*Glycine*	*Glutamine*
CNS	20·14±3·56	27·72±3·61	2·62±0·79	12·04±2·70
Eyes	11·42±3·03	26·82±1·96	3·60±1·33	11·03±5·25
Somites	13·50±1·89	24·73±2·47	3·94±0·66	8·55±1·75
Heart	19·01±2·95	18·17±3·93	4·65±1·30	6·20±2·60
Skin	11·75±2·49	19·88±5·23	3·37±1·26	6·08±2·18
Liver	14·93±3·28	14·44±2·61	3·60±1·60	11·78±3·92
Gut	16·03±2·38	20·73±1·84	4·57±1·58	13·77±1·20
	α-Alanine	*β-Alanine*	*Valine*	*Leucine*
CNS	4·62±2·25	16·71±2·65	4·24±2·02	7·41±2·40
Eyes	8·12±2·66	22·01±6·21	7·44±1·21	9·38±1·58
Somites	4·44±0·98	22·70±5·77	4·23±0·56	5·09±0·62
Heart	6·06±1·80	16·80±4·72	6·20±1·46	21·27±3·90
Skin	8·09±3·06	18·65±6·72	8·98±1·13	23·01±6·37
Liver	5·75±1·14	29·58±9·97	6·34±1·41	10·60±2·58
Gut	6·51±1·96	23·66±1·29	6·14±0·74	5·69±1·10

Values given are means, with standard errors, of 5 experiments.

It will be noted that the heart has a higher total free amino-acid concentration than other parts. This is, however, more an indication of the free amino acid in

the blood-stream than in the heart tissue, since by far the greatest bulk of the so-called 'larval heart' consisted of blood; the heart wall itself was very thin. The content of free amino acid in the blood varies according to the nutritional state of the animal, and the acids are probably simply being transported to supply other tissue proteins.

The liver, apart from its relatively high beta-alanine content, contains less free amino acid than other larval tissues. Knowing the importance of the adult liver in amino-acid metabolism, this is at first surprising: but it is mainly due to remains of yolk in the larval liver. It has been remarked that yolky parts of the embryo contain less free amino acid than other parts, when expressed as concentrations per unit weight. This is simply because yolk is heavy: not because it must be thought metabolically inert.

The free amino acids in the gut necessarily included the products of digestion of its contents, so could not be considered characteristic of gut tissue *per se*.

Other features of the amino-acid concentrations in larval tissues are considered below.

INTERPRETATION OF RESULTS

Although the results reported here do not give a full account of the changes in distribution of free amino acids during development in *Xenopus laevis*, they do show features that are of interest because some interpretation of them is possible, either in terms of the special functions of embryonic regions at the stage of development when they were analysed, or in terms of what larval tissues they are due to form.

(1) *Whole embryos*

A feature of the earliest phases of embryonic development was the high concentrations of free aspartic acid and glutamic acid in the morula, followed by much lower concentrations in the early gastrula. The same sequence of events has been observed in *Rana pipiens* embryos by Kutsky & co-workers (1953), and apparently an analogous situation exists in plant embryos. Certain seeds have been found to contain high concentrations of free aspartic acid and glutamic acid, either in that form or as asparagine and glutamine. These are used up rapidly during the first phases of germination (see Steele, 1934). It is believed that these two amino acids are used as the main nitrogen source by the plant embryo for protein synthesis. As they have fewer carbon atoms per molecule than most other amino acids, and in their amide form, two nitrogen atoms per molecule, they would be an economical nitrogen store. It would not be unreasonable to suppose that in the amphibian embryo, too, the initially large concentrations of aspartic acid and glutamic acid may provide nitrogen for immediate use in the synthesis of new protein. Both these acids have a special importance in protein synthesis because they can be converted into many other amino acids by transamination. In other reactions they link up with carbohydrate metabolism.

which may be of yet more importance to the early embryo, since it uses carbohydrate as its main energy source (Needham, 1950, for review).

(2) *Embryonic regions*

(*a*) *Blastula.* It has been noted that in the blastula aspartic acid is more highly concentrated in the animal half than in the vegetal half. Another known function of aspartic acid is that it participates in forming the pyrimidine ring of nucleotides (Lagerkvist *et al.*, 1951). Its predominance in the animal pole cells may therefore be connected with their more rapid turnover of nucleotide than the slowly dividing vegetal cells.

Thinking of the later fate of regions of the blastula, it is surprising that beta-alanine is the only other amino acid more highly concentrated in the animal half. Part of the animal half is due to form the dorsal lip of the early gastrula—a region characterized by high concentrations of several free amino acids—while the vegetal half, on the other hand, will contribute to the yolky ventral regions of later stages, and these contain less of most amino acids than dorsal parts. One can only assume that a rapid change in the regional concentrations of amino acids must occur at the onset of gastrulation. It would be interesting to know if this change occurs by transportation between regions or by different rates of utilization of the amino acids in different regions.

(*b*) *Early gastrula.* The high total concentration of free amino acid in the dorsal lip is difficult to account for in terms of any one particular function of this tissue in the early gastrula. The lip is known to be an important site both of proteolysis and of protein synthesis (Brachet, 1950). Some of its free amino acids may therefore be the products of protein breakdown, while others may equally well have just been synthesized ready for incorporation into new protein. Decision between these two possibilities cannot be reached without detailed data on exchanges between free and protein-bound amino acid in this region of the embryo from the blastula to the gastrula stage.

(3) *Distribution of individual amino acids*

(*a*) *Aspartic acid, glutamic acid, and glutamine.* These three amino acids show high concentrations in the dorsal lip and have an interesting distribution in later development. First concentrating in the dorsal half of the neurula (aspartic acid does this as early as the neural groove stage) they then become predominant in the brain of the larva. Glutamic acid and glutamine already show a maximal concentration in the head at the tailbud stage. There is no evidence as to whether or not these amino acids pass from the dorsal lip into the presumptive neural plate during gastrulation, as at no stage could the archenteron roof be separated from the neural plate for analysis. It would, however, be most interesting to know if there is any amino acid interchange between these two tissues, during the process of neural induction. The observation in series 2(*c*), that immediately after, but not before, the appearance of the neural groove,

amino-acid differences become noticeable between dorsal and ventral halves of the late gastrula, suggests a connexion between early neural differentiation and the localization of free amino acid.

Even without knowing how aspartic acid, glutamic acid, and glutamine become particularly concentrated in the central nervous system, this finding is of general interest, since brain tissue of a wide variety of animals contains high concentrations of these amino acids, particularly of glutamine (Ansell & Richter, 1954). The roles of glutamine and glutamic acid in brain metabolism have been much discussed recently (see review by Waelsch, 1952). One of their functions is the detoxification of ammonia, which attaches to the glutamic acid molecule, converting it to glutamine. There may be a need for this detoxification process in the dorsal lip of the gastrula, too, since ammonia-production is particularly marked here at the onset of gastrulation (Boell, Needham, & Rogers, 1937). The high concentration of glutamic acid and glutamine in both dorsal lip and brain may, in fact, be better explained by their having similar metabolic needs than by any morphological or chemical continuity between these two tissues during development. It is possible, though, that the two amino acids have later a special role in brain morphogenesis, since they become preferentially concentrated at the anterior end of the embryo just at the stage (tailbud) when the brain is morphologically distinct.

(*b*) *Valine.* This amino acid is more highly concentrated in the dorsal lip than in other parts of the early gastrula. It later becomes concentrated chiefly in the dorsal half of the neurula, then in the dorsal trunk of the hatched larva. This trend in distribution seems clearly related to the developmental fate of the tissues, since the dorsal lip consists of presumptive somites and notochord, both of which become the main constituents of the dorsal trunk of the larva.

Work with metabolic inhibitors suggests that valine has a special importance in the differentiation of somites. Valine analogues prevent the segmentation of somites in the chick embryo (Herrmann, 1953; Rothfels, 1954). In the present data, too, there is evidence that valine is later used up by the developing muscle tissue: for the somites of the late larva contain little free valine. Since mammalian myoglobin contains a high percentage of valine (Block & Bolling, 1951) it may be that the demand for valine becomes particularly marked at the time when this protein is being synthesized in the Amphibian embryo. If so, one has to assume that the mechanism for myoglobin synthesis does not come into action, or is not working efficiently, until quite late in muscle development, to explain the earlier pile-up of free valine in this tissue.

(*c*) *Leucine.* Leucine, like valine, makes up a high proportion of the amino acid in muscle tissue (Block & Bolling, 1951). Moreover, analogues of leucine have the same effect as valine analogues on developing somites of the chick embryo (Herrmann, 1953; Rothfels, 1954). There is, however, no evidence of any accumulation of free leucine in developing somite tissue of *Xenopus.* Leucine does, on the other hand, predominate first in the ventral part of the early

gastrula, then later in the larval skin and heart. Its special significance in the skin is not clear, though there is evidence from the use of leucine analogues on regenerating tail-tips of *Xenopus* larvae (Lehmann & Dettelbach, 1952) that the epidermal cells require leucine for normal migration and mitosis.

(*d*) *Alanines.* Like leucine, alpha-alanine and beta-alanine make up higher percentages of the free amino acid in ventral regions of the early gastrula than in the dorsal lip. Later, alpha-alanine is more highly concentrated in the ventral half of the neurula than in the dorsal half. In the larva the alanines predominate in the eyes, the liver, the heart, and the ventral skin. All these parts of the larva originate from ventral parts of the early gastrula. So it can be said, though with some imprecision since many other tissues form from the same region, that the ventral part of the gastrula shows features in its amino acid content that foreshadow the characteristics of homologous larval tissues.

(*e*) *Glycine.* This is the only amino acid of those studied quantitatively that seemed uniformly distributed in the embryo until the neurula stage; all the others showed some regional differences in concentration in the early gastrula. Possibly the present technique was not sufficiently sensitive to detect early differences in glycine concentration: throughout development there seemed to be very little free glycine compared with other amino acids. This seems worth emphasizing, since radioactive glycine has become a popular tool of experimental embryologists, and one wonders if the results obtained by supplying extraneous glycine to Amphibian embryos bear much relation to normal conditions when there would be very little of this amino acid in free form.

In the neurula and in the tailbud stage, glycine is present in higher percentage in the posterodorsal tissue than in other parts. In the hatched larva, all that is noticeable is a predominance (per unit weight) of glycine in the dorsal half. However, in the older larva it is the somites that contain a higher percentage of glycine than most other tissues: the expected sequel to the distribution in neurula and tailbud stages. It is known, moreover, that adult muscle proteins contain a particularly high percentage of glycine (Block & Bolling, 1951). Here, then, seems to be another instance of a distribution of amino acid in the embryo that foreshadows the amino acid distribution in the protein of homologous adult tissue.

(*f*) *Other amino acids.* One feature of the results reported here that requires explanation is the failure to detect in free form either threonine, phenylalanine, methionine, or histidine: all 'essential' amino acids which, in mammals and birds at least, cannot be synthesized. If the same is true of the amphibian embryo, which has no external source of amino acid, it can only obtain the essential ones by degradation of pre-existing protein. It is therefore surprising that so many of them should never appear in the free amino-acid pool at any stage of development. A specific spray test (Winegard *et al.*, 1948) was used for methionine, with negative results. One may note that other authors have sometimes failed to detect free methionine in Amphibian embryos: Eakin, Berg, & Kutsky (1950) found it

in *Rana* but not in *Hyla*, while Kutsky *et al.* (1953), and Holtfreter, Koszalka, & Miller (1950) were unable to find free methionine in *Rana pipiens* embryos, though they did find phenylalanine, threonine, and histidine. Yet radioactive tracer studies (Eakin *et al.*, 1951; Feldman & Waddington, 1955) indicate that methionine is readily taken up into embryonic protein and may play an important role in morphogenesis. It seems desirable, then, for more attempts to be made to detect small traces of free methionine in the embryo. Histidine, too, may require a more sensitive technique for its detection: ninhydrin certainly is inadequate, as it does not give a colour with quantities less than 8 gamma. Threonine and phenlyalanine give quite intense colours with ninhydrin, however, so it must be concluded that these amino acids were not present in the extracts from *Xenopus* embryos.

It has already been pointed out in the introduction that the composition of the free amino-acid pool is continually being changed by diffusion, active transport, and protein breakdown, as well as by protein synthesis. In the present state of our knowledge, it is a matter of opinion which of these processes one imagines to predominate at any given moment in adult tissue. When considering the differentiation of embryonic tissue, however, it is reasonable to lay particular emphasis on synthesis, since there is immunological evidence that tissue-specific proteins are being formed. In the foregoing discussion changes in the free amino acid have therefore been related as far as possible to synthesis. One has, however, to admit the possibility that the free amino acids in embryonic tissue are not the raw material, but the by-products of metabolism. A hint of this has been given by the distribution of valine: its early accumulation in presumptive somite tissue may have reflected, not so much that it was going to be needed later, as that there was no efficient mechanism for removing it at this stage.

These results do not, then, give incontrovertible evidence that amino acids become distributed in the embryo in regions where they will be in most demand for synthesis of specific proteins. They do, on the other hand, show that even in the early embryo the metabolism of some tissues is sufficiently like that of corresponding adult tissues to result in similar concentrations of free amino acids.

There is still a great need for more systematic studies on amphibian embryos, using radioactive tracers, so that sequences of events in amino-acid metabolism may be followed. Stage-by-stage analyses, even if they are accurately quantitative, do not allow one to make any assumptions about what occurs during the times intervening between the stages analysed. This is shown only too clearly by Kavanau's recent work (1954) on a very close series of stages in the development of the sea-urchin, where he found many more fluctuations in amino-acid concentration than had been thought to occur from observations on more widely separated stages. Chemical analyses can furnish only the framework for an account of amino-acid metabolism in the embryo: the gaps have still to be filled by *in vivo* experimentation.

SUMMARY

1. Free amino acids in embryos and larvae of *Xenopus laevis* have been studied chromatographically. Quantitative measurements have been made on eight amino acids, and their concentrations have been compared between different regions of the embryo and between different tissues of the larva.

2. Extracts of whole embryos showed higher concentrations of free aspartic acid and glutamic acid in the morula than in the early gastrula; significantly more leucine in the late gastrula than in the early gastrula, and less glutamine in the neurula than in the early tailbud.

3. A number of free amino acids were more highly concentrated in the dorsal lip than in other parts of the early gastrula. Differences were also apparent between dorsal and ventral halves of the neurula, early tailbud stage, and hatched larva. In the early tailbud stage there was more glutamine in the head than in other regions and a higher percentage of glycine in the trunk.

4. The main features of the free amino-acid concentrations in larval brain, eyes, somites, heart, liver, and skin are described and compared. In some features the free amino-acid concentrations in embryonic tissues resemble those of the larval tissues that derive from them in development.

5. The evidence for special functions of certain amino acids in morphogenesis and in the synthesis of specific proteins is discussed.

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EXPLANATION OF PLATE

Chromatograms of the free amino acids in whole embryos. Each represents material extracted from fifty embryos.

FIG. A. Morula.

FIG. B. Blastula.

FIG. C. Early gastrula.

FIG. D. Late gastrula.

FIG. E. Neurula.

FIG. F. Tailbud.

Key to spots numbered: 1. Glutathione. 2. Aspartic acid. 3. Glutamic acid. 4. Glycine. 5. Glutamine. 6. Lysine. 7. Alpha-alanine. 8. Beta-alanine. 9. Valine. 10. Leucine. 11 and 11*a*. Cystine.

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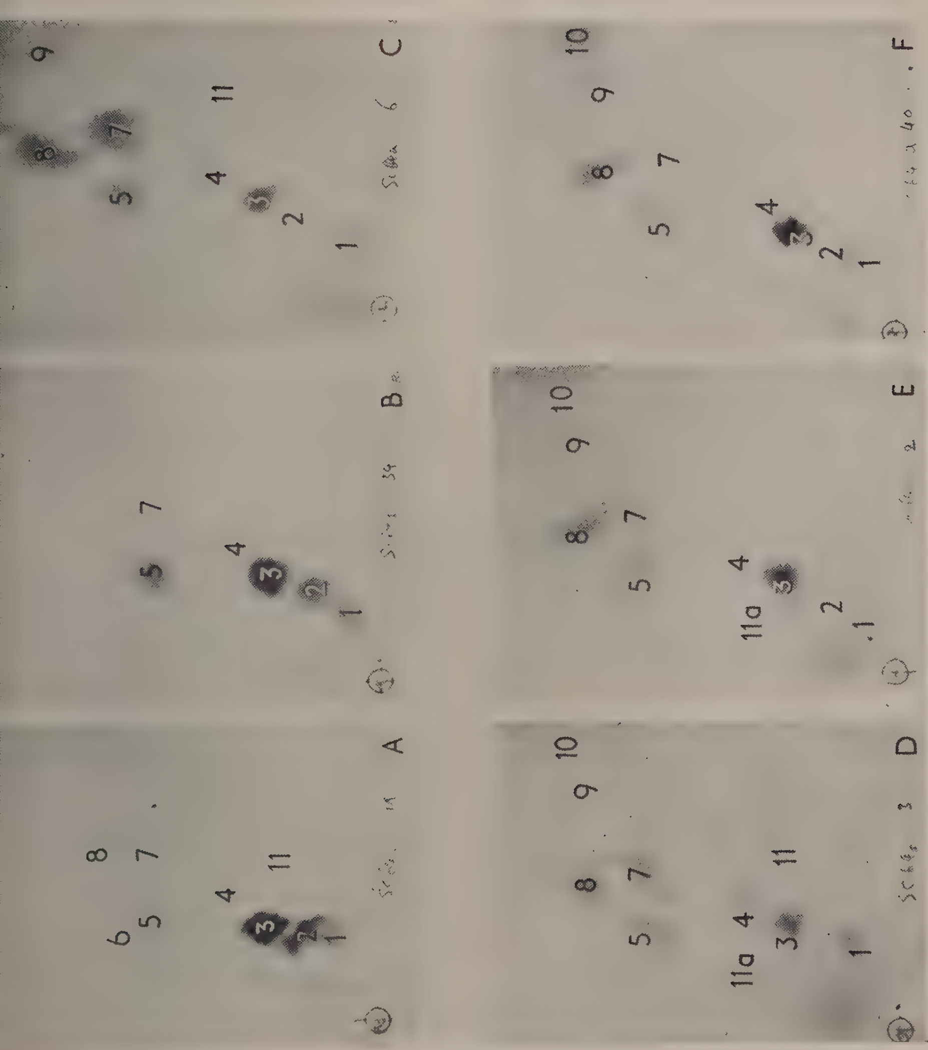

E. M. DEUCHAR

Plate

Development of Embryonic Mouse Gonads Transferred to the Spleen: Effects of Transplantation Combined with Genotypic Autonomy

by ELIZABETH S. RUSSELL, LOUISE M. MURRAY, ELEANOR M. SMALL, *and* WILLYS K. SILVERS[1]

From the Roscoe B. Jackson Memorial Laboratory, Bar Harbor

WITH TWO PLATES

INTRODUCTION

KNOWLEDGE of the time and manner of origin of genetically induced defects may well be sought by classical methods of experimental embryology, involving transplantations between genotypes, providing the effects on a tissue of residence in a host of different genotype is not obscured by generalized reactions to transplantation. Such experimental transplantation is reported here, applied to analysis of the pleiotropic effects of the deleterious alleles at the *W*-locus in the mouse (Russell, review, 1955).

Through extensive investigation it has become apparent that the three major types of defect associated with these alleles are already established at birth. In contrast to their normal (*ww*) littermates, new-borns of the genotypes with which this paper will largely be concerned (W^vW^v and *WW*) are severely anaemic (Russell & Fondal, 1951), their gonads are almost totally devoid of germ-cells (Coulombre & Russell, 1954), and their hair follicles lack melanoblasts (Silvers, 1953). It is further known that these same genotypes are deficient in blood-forming capacity at least as early as 12½ days of embryonic life (Borghese, 1952; Attfield, 1951). The erythrocyte levels of both W^vW^v and *WW* are measurably lower than those of littermates at the 14th day of embryonic life (Russell, Fondal, & Smith, 1950). Conventional histological methods have not given critical evidence concerning the relative numbers of germ-cells in normal *ww* and potentially sterile W^vW^v and *WW* at 12 days of embryonic life, although differences have been observed in 16-day embryos.

One reasonable hypothesis for the anaemia-germ-cell pleiotropic effects of these genes attributes the lack of germ-cells in ovaries and testes of new-born *WW* and W^vW^v individuals to a suppression of their pre-natal development or

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survival caused by the anaemia present in the same embryos. It is essential to this theory that anaemia be present before the germ-cell defect has reached an irreversible maximum expression.

A second possible hypothesis suggests that the germ-cell defect develops by local gene-action completely independent of the embryonic anaemia. Time of first appearance and final determination of each defect is, of course, less essential to this interpretation.

These opposing theories may be susceptible to test by observations of the nature of development of germ-cells in gonads from embryos of normal and defective genotypes, transplanted at stages prior to final determination, to a neutral site with a rich blood-supply. One necessity for such analysis is a site which sustains development sufficiently normally to allow interpretation of genotypic differences. Certainty of interpretation will also be greatly enhanced if identification of genotype of embryos can be made at the time of operation. With methods available for this study, the earliest stage at which *W*-genotype identification could be attempted from observation of living embryos was $12\frac{1}{2}$ days. The present paper is a report of observations of gonads from new-borns and 12–16-day embryos transplanted to the spleen of normal adult castrate male hosts.

MATERIAL AND METHOD

Choice of host site

The receiving tissue must have a rich blood-supply, must be very different from the gonad in histology, so that all implanted tissue can be recognized, and must be capable of supporting reasonably normal development of gonads of both sexes. Since the spleen has a rich blood-supply and is completely different in its histology from any gonadal tissue, it presents many advantages as a transplant site. Spleens of castrate mice have been used extensively as host sites for adult ovaries (Furth & Sobel, 1947; Li & Gardner, 1947; Klein, 1952; Hummel, 1954; and Gardner, 1955), largely because long periods of splenic residence lead to ovarian tumour formation. Ovarian function is maintained on retransplantation to the ovarian capsule, however, after short periods of splenic residence (Little, Hummel, Eddy, & Rupple, 1951). Ovaries of new-born mice transplanted to castrate spleen become abnormal after prolonged residence, with defects first observed after 4 weeks (Guthrie, 1954, 1955). The evidence of alteration of ovaries made imperative the selection of a short term for the experiment, and tests of the effects of short terms of splenic residence upon the development of ovaries and testes of normal new-borns. Preliminary trials of the effects of 2 weeks' splenic residence on gonads taken from normal new-borns indicated that although transplant to the spleen for 2 weeks caused some reduction in the spermatogenic activity of the testis, otherwise normal development was obtained in both sexes.

For all subsequent experiments the spleen of 4–8 week C57BL/6 males, castrated 1–2 weeks before the operation, was selected as host site.

Choice of donors

All donor animals were embryos and new-borns of the various genotypes segregating from $W^vw \times W^vw$ and $Ww \times Ww$ crosses. They were isogenic with C57BL/6, and thus acceptable to the selected hosts. In all cases, identification of donor genotype was made at the time of operation rather than on histological study, to avoid possibility of circular reasoning.

The age of embryos was determined within 8 hours by plugs from timed matings, and postpartum matings were avoided. In operations using new-borns and 16-day embryos, normal and W^vW^v or WW genotypes could be distinguished by inspection (W^vW^v and WW appear much paler), and sex could be determined at the time of operation by position and shape of gonads. In 14-day embryos it was not always possible to establish the sex of transferred gonads at the time of operation, and at 12 and 13 days no attempt at such identification was made. Hence sex of gonads transferred at early stages could only be inferred from results.

The genotype was difficult to determine in 12–13-day embryos. In 13-day embryo litters segregating for WW, slightly less than $\frac{1}{4}$ (11 of 52) were recorded at operation as having noticeably pale livers. It was assumed these were of the extreme anaemic WW genotype. All of the pale-liver embryos found in any uterus were used as donors, along with an equal number of presumed *ww* or *Ww* donors, selected at random from among the embryos with bright red livers. The *W* genotype of embryos in crosses segregating for W^v/w were identified with the help of a linked gene, *lx*, whose phenotype has been described as early as the 12th day of embryonic life (Carter, 1954). Both W^v and *lx* are on the third chromosome, 18 units apart (Carter, 1951*b*; Russell, unpublished data). An isogenic strain carrying both of these genes on one third chromosome was developed by repeated backcrosses of the double heterozygote to C57BL/6. On this C57BL/6 background practically all *Lxlx* adults have extra and frequently misshapen digits on the inner side of one or both rear feet, and all *lxlx* individuals have much more extreme abnormalities of hind legs and feet (Carter, 1951*a*). In $12\frac{1}{2}$–13-day embryos Carter (1954) has described a narrowing of the leg combined with an overgrowth of the inner side of the rear feet in presumed homozygous *lxlx*, and a slight enlargement of the inner side of the rear feet of *Lxlx* heterozygotes. In $11\frac{1}{2}$–12-day embryos Carter's clearest evidence of homozygosity for *lxlx* was a narrowing of the rear leg, and change of its angle with the body although he also observed some enlargement of the inner side of the feet. One hundred and eight 13-day embryos from matings of W^vwLxlx parents classified according to the presence and extent of abnormality of the rear feet gave 32 completely normal (presumed *LxLx*), 48 with mild abnormalities of one foot (presumed *Lxlx*), 6 with mild to marked abnormalities of both feet (doubt-

ful, either *LxLx* or *lxlx*), and 22 with extreme abnormalities of both feet (presumed *lxlx*), a reasonable approximation of the expected 1 : 2 : 1 ratio. Recognition of the 12-day *lxlx* phenotype was less certain. Eight of 54 12-day embryos from $W^v wLxlx$ matings were classified as *lxlx* on the basis of narrowing of the leg and abnormal shape of the foot. Since in these crosses approximately 80 per cent. of individuals homozygous for *lxlx* are also homozygous for W^vW^v, the appearance of rear feet and legs offers the best method at present available for distinguishing normal and potentially sterile embryos at the time of operation. Crossovers and difficulty in *lxlx* identification are recognized as possible sources of error. From any one uterus all of the presumed W^vW^v embryos and an equal number of normal embryos were selected as donors. Wherever possible, the normals had completely normal feet (*wwLxLx*) but $W^v wLxlx$ donors were occasionally used when *ww* embryos were not available in the litter.

Technique of operation

Pregnant females were anaesthetized with nembutal and the uterus exposed. Beginning at the anterior end of the uterus, the section containing a single embryo was removed, the uterine wall and embryonic membranes cut, and the exposed embryo transferred to 0·85 per cent. saline solution for examination of feet and liver colour. Those selected as donors were spread on filter paper with the ventral surface exposed. After removal of the ventral abdominal organs, the gonads, usually with some adjacent tissue, were placed into the tip of a No. 19 hypodermic needle with a plunger inserted to make it serve as a trocar. The host was anaesthetized, its spleen temporarily exteriorized, a nick cut in its surface with scissors, and the donor tissue extruded from the needle-trocar into the body of the spleen.

Technique of histological observation

The transplanted gonads were allowed to grow in the spleen to the time equivalent to 14 days post-natal (14 days for new-borns, 21 days for 13-day embryos). The hosts were then sacrificed and the spleen examined. In many cases the region of implanted tissue was externally visible; such regions were saved and sectioned serially. If no implant was visible, the entire spleen was sectioned serially. Examination of histological sections formed the basis for quantitative and qualitative evaluation of the results of these gonad transfers. All types of tissue recognizable in the implants were recorded for each case.

If ovarian tissue was observed, the total number of follicles was established by examination of all sections, with counts of nuclei of ova. The stage of development of the most advanced follicles found in each transplant was also recorded.

If testicular tubules were observed, the proportion containing spermatogenic cells was tested by Chalkley sampling (Chalkley, 1943). One section in each slide row (approximately 5 per cent. of all sections) was classified. In each test-section the tubules falling under four pointers were classified for presence or absence of spermatogenic cells. No detailed attempt was made to classify stages of spermato-

genesis observed, nor to compare relative numbers of seminiferous cells per functioning tubule section. To avoid sampling error arising from variability in the number of transplanted tubules observed, the index of spermatogenic activity for each genotype was based on proportion of functioning tubule-sections in a combination of all pertinent testis transplants rather than on presence or absence of functioning tubules in a given implant.

RESULTS

Success of operation

Variables other than genotype of the donor contributed considerably to chance of recovery of pertinent tissue. The chance of recovering any transplanted tissue varied with the age of the donor at transfer. Tissue implants were found in 77 per cent. (153/200) of the recipient spleens, but the percentage was lower

TABLE 1

Tissue types recovered from spleen following gonad transplant operations

Stage operated	*New-born*	*16-day embryo*	*14-day embryo*	*13-day embryo*	*12-day embryo*
Total numbers	42	18	28	80	32
No implant	9*	1	4	22	11
Testis	10	7	12	36	7
Ovary	17	6	4	8	1
Ducts with ovary	12	5	4	4	1
,, ,, testis	2	0	1	7	0
,, without gonad	7*	3	2	3	0
Kidney	0	4	6	27	11
Intestine	0	3	0	3	1
Skin	0	0	1	2	3
Bone (and marrow)	0	0	5	5	5
Cartilage	0	0	1	1	4
Muscle	1	0	0	1	4

* Classified as ovary at transplantation.

with 12-day donors (21/32 or 66 per cent.) than with 16-day and new-born donors (50/60 or 83 per cent.). Other types of tissue were found in many implants (Table 1), including kidneys with glomeruli and collecting tubules, intestinal epithelium, skin (often with hair follicles), bone and included marrow, and muscle. As would be expected from the position of the transferred organs, kidney-tissue appeared very frequently, especially when donors were of ages (12 and 13 days) when gonad and kidney are very closely associated. Other extraneous tissue types (muscle, skin, bone, cartilage) were especially frequent in transfers from early stages. Large ducts or cysts were frequently found, more commonly with ovaries than with testes. Their appearance was suggestive of oviducts. The probability of recovering gonad tissue also varied with the developmental stage of the donor. In transfers from 12- to 14-day embryos, testes, all showing excellent differentiation of tubular structure, were recovered much

more frequently than ovaries. A probable explanation, in line with previous reports of operations involving embryonic gonads (Everett, 1943) is that the non-germ-cell tissue of embryonic testes survives better in the spleen than does that of ovaries. In those cases where female germ-cells from ovaries of fertile genotypes were recovered, they had differentiated more normally in the spleen than had male germ-cells in transplanted testes. When recovered after 18–22 days in the spleen, ovaries from 16- to 12-day normal ovaries (Plate 2, fig. 5) contained many follicles, all of which had developed to stages corresponding to those seen in a normal 14-day post-natal ovary (Plate 1, fig. 1) (Coulombre & Russell, 1954). By contrast, the number of spermatogenic cells in functioning tubules in a testicular transplant never approached the level in the normal 14-day post-natal testis. In place of the typical 4–5 layers of seminiferous cells, with numerous primary and secondary spermatocytes and meiotic division plates (Plate 1, fig. 2) tubules found in transplants usually showed a single incomplete layer of cells, spermatogonia and/or spermatocytes (Plate 2, fig. 6). Testicular tubules survive easily, but their spermatogenesis is diminished in the spleen.

Differences associated with genotype

The gonads of 14-day post-natal *ww* individuals (Plate 1, figs. 1, 2) are strikingly different in appearance and germ-cell content from those of 14-day W^vW^v individuals (Plate 1, figs. 3, 4). Regardless of the developmental stage at transfer, ovaries from potentially fertile genotypes (*ww*, W^vw, *Ww*) recovered

TABLE 2

Results of 2 weeks' residence in the spleen of normal (ww) *and anaemic* (W^vW^v) *castrate hosts on germ-cell numbers in gonads transplanted from new-born normal* (ww) *and anaemic* (W^vW^v *or* WW) *donors*

	Ovaries			*Testes*		
Genotypic combination of donor and host	*Number of operations*	*Gonads recovered*	*Number of follicles*	*Number of operations*	*Gonads recovered*	*Ratio active total tubules*
ww in *ww* . . .	15	9	175, 155, 132, 117, 104, 45, 41, 24, 9	6	6	253/425
ww in W^vW^v . .	10	6	138, 124, 70, 67, 19, 12	2	2	64/85
W^vW^v in *ww* . .	6	1	0	2	2	65/201
WW in *ww* . .	1	1	0			

from the spleen at the post operative time equivalent of 14-days post-natal contained more ova than did ovaries from potentially sterile genotypes (*WW*, W^vW^v).

In a series of transfers of ovaries from normal and anaemic new-borns (Table 2) ovarian tissue was recovered from 9 of 15 *ww* ovaries after 2 weeks'

residence in the spleen of adult castrate *ww* males. All contained large numbers of follicles, the most advanced showing a many layered granulosa, frequently with coalescing antra (stages 5 and 6, Coulombre & Russell, 1954). Ovarian tissue was recovered from only 1 of 6 W^vW^v new-born ovaries transferred to the spleen of *ww*, and this one lacked ovarian follicles. It is interesting to note that 6 of 10 ovaries transferred from normal *ww* new-borns into anaemic W^vW^v castrate male spleens developed normally, all with large numbers of follicles, the most advanced at stage 5 or 6. Although it was more difficult to obtain successful ovarian transplants from early (12–13-day) embryo donors, in those cases where

TABLE 3

Results of transfers to the spleen of gonads from 12- to 13-day embryos of genotypes identified at time of transfer as normal (ww *or* Ww) *or severely anaemic* (WW *or* W^vW^v). *Hosts were adult castrate* ww *males. All tissues were recovered at the stage equivalent to 14 days post-natal, and sex of implanted gonads determined from histological study of recovered tissue*

Age in transfer	*Number operated*	*Testes recovered* Genotype	*ww*	W^vW^v	*WW*	*Ovaries recovered* Genotype	*ww*	W^vW^v	*WW*
13 days	56	Number of gonads	8	9	4	Number of gonads	5	2	1
		Proportion of active tubules	256/338	44/373	10/163	Total follicles per implant	118, 18, 16, 15, 13	0, 3	0
12 days	17	Number of gonads	4	2		Number of gonads	1		
		Proportion of active tubules	239/274	9/45		Total follicles per implant	29		

ovarian tissue was recovered the number of ova was higher in transfers from potentially fertile genotypes (Table 3; Plate 2, figs. 5, 7). The numbers of follicles observed in ovaries recovered from transfers of 13-day normals approximated numbers recovered from transfers of new-born normal ovaries (Table 3). The most advanced follicles observed were at stages 5 and 6.

In testicular transplants, the proportion of functioning tubules was uniformly higher in transplants from potentially fertile than from potentially sterile genotypes. Although in transfers from normal genotypes residence in the spleen inhibited spermatogenesis to some extent, this was not sufficient to prevent expression of the genotypic difference. All of six testes from *ww* new-borns transferred to castrate *ww* spleens were recovered 14 days later (Table 2). Slightly more than half of the tubules (253/425) showed spermatogenic cells. Two testes were transferred to the spleen W^vW^v castrates, where they developed well, showing a high proportion (64/85) of tubules with spermatogenesis. The proportion of such tubules was much lower (65/201) in two transplants of testes from W^vW^v

new-borns. Similar differences between implants from normal and potentially sterile donors were found with transplants made at early embryonic stages (Table 3; Plate 2, figs. 6, 8).

The proportion of tubules with spermatogenesis was high in 8 testes recovered from transfers from 13-day *ww* embryos (256/338) and in 4 testes recovered from transfers from 12-day *ww* embryos (239/274). The proportion was much lower in 9 testes recovered from W^vW^v 13-day embryos (44/373), in 2 from W^vW^v 12-day embryos (9/45), and in 4 from *WW* 13-day embryos (10/163). Similarly, the total number of follicles was much higher in 5 ovaries recovered from transfers from 13-day *ww* embryos (118–13) and in one ovary from transfers from 12-day *ww* embryos (29) than in 2 ovaries recovered from transfers from W^vW^v 13-day embryos (0, 3) or in one ovary recovered from transfer from a 13-day *WW* embryo (0). These latter ovarian transplants were identified as ovarian by the swirling arrangement of stromal cords.

Thus it appears that explantation of gonads from a potentially anaemic sterile genotype to a site with a rich blood-supply at mid-embryonic stages does not alter the genotypic pattern of germ-cell development. In the age range tested, from the 12th day of embryonic life to birth, the time of removal from the natural (anaemic) milieu and transfer to the abundant blood-supply provided in the host spleen had no effect on the degree of germ-cell deficiency observed in gonads of either sex.

DISCUSSION

In all analyses of pleiotropism or studies of gene-action involving transplantation between differently affected genotypes or explants to tissue culture, possible non-specific effects of the operations involved must be taken into consideration. Hence it has appeared pertinent in this study to present evidence on effects of the operation and of splenic residence on subsequent development of embryonic gonads. From the results it appears that the trauma of operation has reduced the chance of recovering ovarian tissue, but has not seriously affected differentiation in successful transplants. In contrast, testicular tubules appear to have survived well in the spleen, but the multiplication and/or differentiation of their contained spermatogenic cells was somewhat inhibited in this foreign milieu.

In spite of these limitations, it is clear that in transplants of both ovaries and testes there are fewer germ-cells if the donor is of the potentially sterile *WW* or W^vW^v genotype than if it is of the normal fertile (*ww*) genotype. This agrees with the findings of Borghese (1955 and personal communication) who explanted tissues from 12-day embryos from $Ww \times Ww$ matings into tissue culture and observed genotypic autonomy of development. These findings are also in complete accord with the report of Mintz & Russell (1955) that the numbers of primordial germ-cells (not yet in the gonad) found in *W*-series defective genotypes at the 11th day of embryonic life correspond to the known differences in numbers of gonadal germ-cells at birth in the same *W*-series genotypes.

Thus this analysis of pleiotropic relations by means of transplantation between genotypes has been one of several sources of information indicating that the germ-cell defect in W^vW^v and *WW* genotypes has reached full expression at the earliest stage ($12\frac{1}{2}$-day embryo) at which evidences of blood-forming defect have been identified in the same genotypes.

Since no gonads were transplanted from defective genotypes at stages prior to the full development of their germ-cell abnormality, no critical test of the alternative hypotheses suggested in the introduction has been possible. However, the time of possible influence of embryonic anaemia on initial development of germ-cell defect has been limited to the period before the 12th day of embryonic life.

The inter-genotype transfers have, nevertheless, contributed other important information. The extremely small numbers of germ-cells observed in splenic transplants of gonads from *WW* and W^vW^v embryos after 18–22 days in *ww* adult hosts indicates no differential influence favouring increased proliferations of germ-cells during this period, resulting from differences in blood-level of surrounding tissues, or from any other differences associated with *W*-series genotypes.

SUMMARY

1. Certain aspects of the pleiotropic relationship between the embryonic anaemia and germ-cell defect produced by deleterious alleles of the *W*-series in the mouse were tested by transfer of gonads from new-borns and 12–16-day embryos into a neutral site with rich blood-supply, the spleen of adult castrates.

2. Splenic residence cut down the recovery of ovarian tissue from transplants at early stages, and slightly inhibited the amount of spermatogenesis at all stages.

3. In spite of such environmental limitations, it was clear that at all stages transplants of both ovaries and testes contained fewer germ-cells at the 14-day post-natal stage if the donor were of defective sterile genotype than if it were of normal fertile type.

4. This supports the hypothesis that genotypic autonomy of germ-cell level is established by the 12th day of embryonic life, and further development is independent of the *W*-genotype of surrounding tissue. The implications of these findings for understanding of pleiotropic relations are discussed.

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EXPLANATION OF PLATES

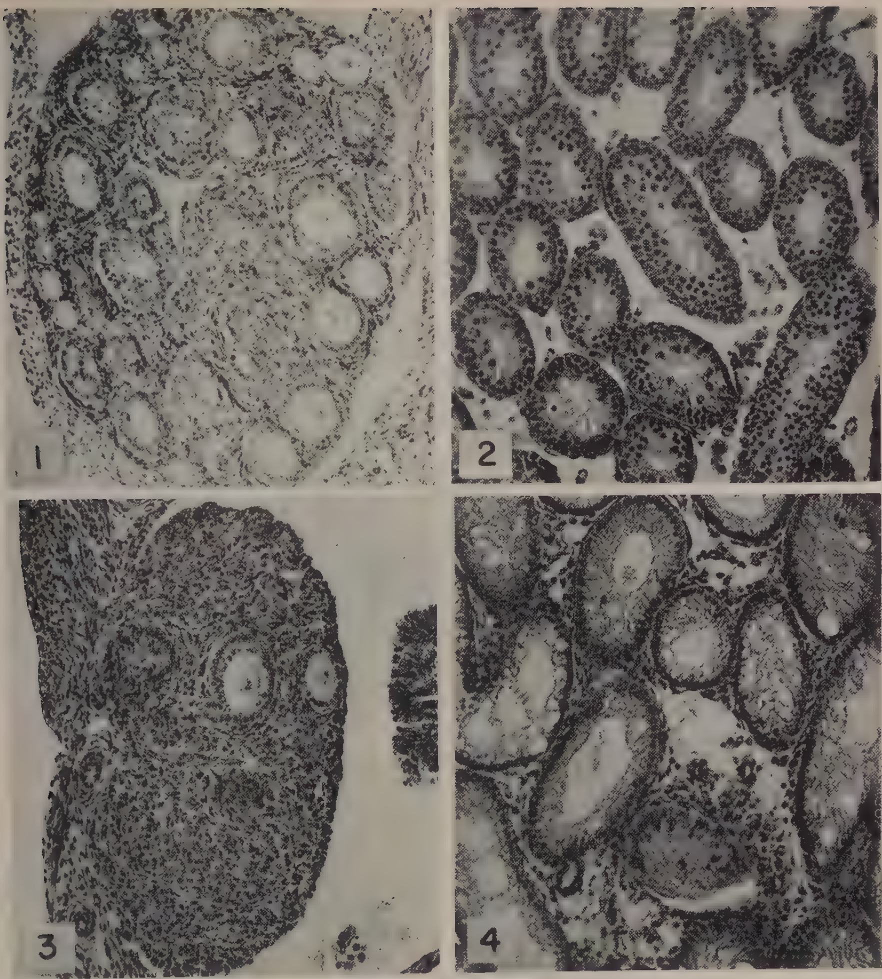
PLATE 1

FIG. 1. Cross-section, ovary from 14-day *ww* female. Most advanced follicle stage 5, many-layered granulosa. ×150.

FIG. 2. Cross-section, testicular tubules from 14-day *ww* male. Dark staining points are spermatogenic cells. ×150.

FIG. 3. Cross-section, ovary from 14-day W^vW^v female. ×150.

FIG. 4. Cross-section, testicular tubules from 14-day W^vW^v male. ×150.



E. S. RUSSELL, L. M. MURRAY, E. M. SMALL, *and* W. K. SILVERS

Plate 1

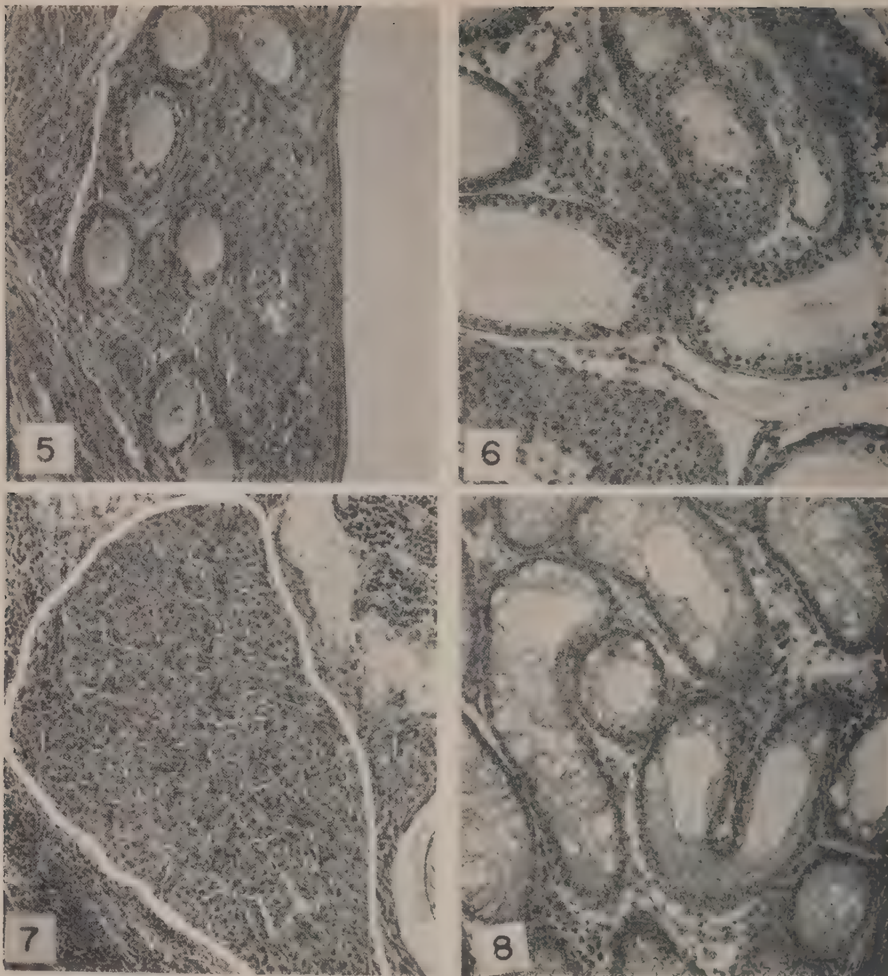


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Plate 2

PLATE 2

FIG. 5. Cross-section, *ww* ovary recovered after 21 days in the spleen. The ovary was transplanted from a 13-day embryo. $\times 150$.

FIG. 6. Cross-section, *ww* testicular tubules recovered after 21 days in the spleen. The testis was transplanted from a 13-day embryo. $\times 150$.

FIG. 7. Cross-section, W^vW^v ovarian tissue recovered after 21 days in the spleen. The ovary was transplanted from a 13-day embryo. Ovarian tissue, consisting of stromal cords, is outlined in white. $\times 150$.

FIG. 8. Cross-section, W^vW^v testicular tubules recovered after 21 days in the spleen. The donor was a 13-day embryo. $\times 150$.

(Manuscript received 13:ii:56)

On the Neural Crest of the Lamprey Embryo

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WITH TWO PLATES

INTRODUCTION

THE neural crest is an embryonic tissue found only in craniate vertebrates, but important in the embryogenesis of all of them in which it has been studied. It may be assumed to be of great antiquity and, indeed, to have been present in the latest common ancestors of cyclostomes and gnathostomes, its acquisition having been an early and critical step in the evolution of the craniates. It follows that its properties in living cyclostomes have a twofold interest. It is possible that it is here in one or more ways 'primitive', and that its study will reveal something of an early stage in the evolution of the vertebrate embryo. On the other hand, the presumptive fate of the crest in cyclostomes may differ from that of the crest in higher vertebrates for another reason. For certain structures in gnathostomes that are of crest origin, in particular elements of the cartilaginous visceral skeleton, are only doubtfully homologous with their cyclostome analogues. Thus should the cranial neural crest in cyclostomes prove to be non-skeletogenous this might be a reflection either of its own primitiveness or of the different evolutionary origins and morphological status of the visceral skeleton in agnathans and gnathostomes.

Since Koltzoff (1901) first recognized the neural crest in the lamprey embryo, and attributed to it much of the mesenchyme of the head, a number of workers, using histological methods of study, have been concerned with the problems it presents. Their findings are summarized in the important monograph of Damas (1944). Briefly it may be said that while most of them accept the existence of an extensive mesenchyme of ectodermal origin in the head, there has been less agreement on the relative contributions made to it by the neural crest and by placodes. Further, there has been little agreement on the problem of whether the branchial basket and the trabeculae cranii (anterior parachordals of Sewertzoff and de Beer) are, wholly or partly, of ectomesenchymal origin and, if they are, from which kind of ectomesenchyme, neural crest or placodal, they are derived. In

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the case of the so-called trabeculae cranii the problem is still further complicated by the possible dual origin of these elements which may, as Johnels (1948) thinks probable, be formed from paraxial mesoderm posteriorly and from ectomesenchyme anteriorly as are, of course, the trabeculae cranii of amphibians.

Experiments involving the extirpation of small lengths of the pre-migratory neural crest of lamprey neurulae and their homoplastic transplantation to the flank (Newth, 1951) suggested that it gave rise to melanophores, elements of the dorsal root ganglia of the head, and to mesenchyme, but failed to show any morphogenetic connexion between crest and head skeleton. This result was suspect, however, for two reasons. In the first place regulation, in the form of migration of crest cells forwards and backwards into the wound area, might prevent small deficiencies in the pre-migratory crest from causing corresponding defects in the skeleton. In the second place grafts of pre-migratory crest to the flank might fail to follow their presumptive fate, if this were in part a skeletogenous one, because of the absence in the trunk of some influence, normally available in the head, promoting chondrification and necessary for it. This second objection received support from experiments on urodeles (Newth, 1954) which confirmed the opinion of Hörstadius & Sellman (1946) that in these animals chondrification of head crest cells will not necessarily occur if they are grafted to the flank before migration begins.

Further experiments on the lamprey neural crest, designed primarily to determine with greater certainty its contributions to the tissues of the head, are described in this paper. The results of some, but not all, of them have been briefly reported in Newth (1955).

MATERIALS AND METHODS

The lamprey material used in these experiments was, in most cases, obtained by allowing specimens of *Lampetra planeri*, taken from their nests, to spawn in sinks in the laboratory. In a few cases ripe males and females were stripped. Two series used embryos of *L. fluviatilis* obtained from stripped adults. The xenoplastic implantations were made with neurulae of *Triturus cristatus* or *T. helveticus* as hosts.

Decapsulation, operation, and wound-healing took place in full-strength Holtfreter solution, subsequent culture until hatching stages in Holtfreter solution diluted ten times. To both these fluids 1 gm. of sulphadiazine sodium was added for every 400 ml. After hatching stages had been reached the larvae were reared in tank water until old enough for histological examination. They were then killed in Bouin's fluid, sectioned at 6 or 8 μ, and the sections stained in Ehrlich's haematoxylin and eosin.

In the description of the experiments which follows a distinction is made between anterior and posterior cranial neural crest. This distinction is, in fact, between that part of the crest which would be expected to give rise to the trabeculae cranii (anterior parachordals) and that part which would give rise to the

branchial basket cartilages were either of these structures derived from the crest. The extents of the two regions are shown in Text-figs. 9 and 10.

RESULTS

I. *The homoplastic transplantation of head tissues to the flank*

In the experiments reported earlier (Newth, 1951) cranial neural crest transplanted to the flank homoplastically failed to form cartilage. The hosts were, however, killed at an age when their own head skeletons had only just begun to chondrify. It seemed possible that a longer life might permit the grafts to show an ability to form cartilage in this situation, but rather later than in normal development. Accordingly these experiments were repeated, and, in addition, two other groups of head tissues were tested for their capacity to chondrify in the trunk. The first was the lateral wall of the pharynx *before* its colonization by migrating crest cells, and the second was the same region *after* this migration was complete.

(*a*) *Anterior and posterior cranial neural crest*

Small pieces of anterior and posterior cranial neural crest were transplanted homoplastically to the flank (see Text-figs. 1 and 2). The hosts were then cultured for as long as possible which means, in effect, until some time after the disappear-

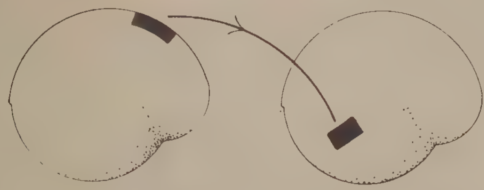
TEXT-FIG. 1. Homoplastic transplantation of anterior cranial neural crest to the flank.

ance of their yolk reserves at an age of about 45 days when they have reached a total length of nearly 8 mm. Relatively few experimental animals lived so long, and only eleven hosts (three bearing grafts of anterior cranial crest and eight with posterior) by reaching this stage were deemed worthy of histological study.

To outside appearance their development had corresponded to that of the animals in the earlier series. The grafts early became the centres of intense concentrations of melanophores, though, as the hosts' own pigmentation developed, it ceased to be possible to identify those melanophores at some distance from the graft site as being either of host or of donor origin. The graft itself remained much more densely pigmented than any other part of the flank.

Histological examination showed that the grafts had been alive and healthy until the death of the hosts, but they differed from those of the earlier series only

in the level of histogenesis achieved. Typically, a small neural vesicle, with clearly differentiated grey and white matter, lay surrounded by a sparse popula-

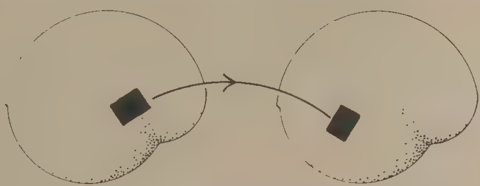


TEXT-FIG. 2. Homoplastic transplantation of posterior cranial neural crest to the flank.

tion of mesenchyme cells and melanophores as shown in Plate 1, fig. D. In no case was cartilage present in or near the graft. Grafts of anterior and of posterior cranial crest did not differ.

(*b*) *The pharynx wall*

Now if it were true that the neural crest played no part in the formation of the branchial basket we might expect grafts of the lateral wall of the pharynx, taken before the ventral migration of the crest had begun, to form cartilage when transplanted to the flank. This possibility was tested in the experiment shown in



TEXT-FIG. 3. Transplantation of the wall of the pharynx, before its invasion by neural crest cells, to the flank.

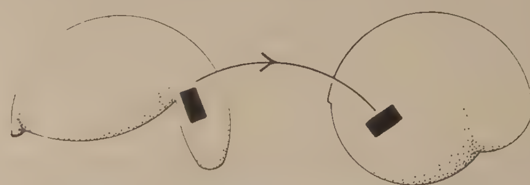
Text-fig. 3. The hosts were cultured until they had passed the age at which their own branchial baskets had started to chondrify, i.e. until they were more than 6·8 mm. in total length. They were then killed and examined histologically.

It was clear from observation of the living animals that the grafts were being extraordinarily well assimilated into the flanks of their hosts. It was usually possible to identify the boundaries of the graft for 1 or 2 days after operation, but thereafter it was impossible. Sections of the hosts showed that this appearance was not deceptive since of the sixty-one animals studied the graft could be identified with certainty in the sections in less than half a dozen, and in these the graft region was only betrayed by small differences in the yolk-platelet complement of the endoderm cells. In particular there was no trace of gill pouch forma-

tion and the gut-wall was separated from the epidermis by the lateral plate mesoderm, its coelom, and the ventral parts of the myotomes throughout the whole length of the trunk.

Only in one animal was an ectopic cartilage found. This was a small rod near the anus, and its significance will be discussed below.

The almost total failure of these grafts or the complete failure of crest grafts to give cartilage in the flank might be due to an influence there which inhibited chondrification, or to unforeseen consequences of the experimental technique used. A further series of grafts to the flank was therefore made, using as donors older embryos in which the colonization of the head by the ventrally migrating crest cells was complete. From them were taken small pieces of the lateral wall of the pharynx which were then grafted to the flank of neurulae (see Text-fig. 4).



TEXT-FIG. 4. Transplantation of the wall of the pharynx, after its invasion by neural crest cells, to the flank.

Such grafts would, of course, contain branchial endoderm, ectoderm, mesoderm, and cells of crest origin. As before, the hosts were allowed to live until they were longer than 6·8 mm. and then killed and sectioned.

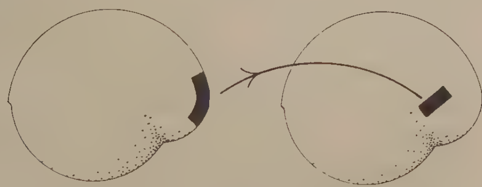
These grafts did not assimilate well into the hosts' flanks. They remained visible as small protuberances which were easy to identify in the whole animal or in section. Of the first six animals sectioned five had rods or nodules of cartilage associated with the grafts. This was regarded as a sufficient answer and further animals were not examined histologically. It follows, therefore, that while the trunk may lack influences promoting the chondrification of head tissues that are normally skeletogenous, the environment it provides is not actually inimical to chondrification as such.

This result makes it possible that the exceptional animal of the earlier series owed its cartilage to operative error, and that the donor was poorly staged as a young neurula when, in fact, its crest had already begun to migrate. The inclusion of even a few crest cells in the dorsal part of the graft would, of course, invalidate the experiment.

II. *The homoplastic transplantation of cranial neural crest to the head*

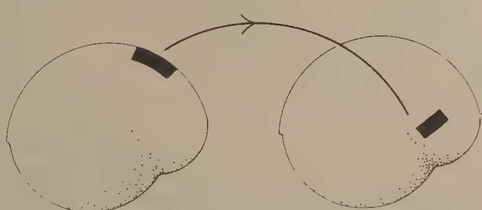
While amphibian experience suggests that transplantation to the flank is not a satisfactory test for the capacity of the cranial crest to form cartilage, it also throws doubt on the value of transplantation to the head for this purpose. For, in

Amphibia, an excess of head crest cells does not necessarily lead to the formation of more cartilage than is normal, there being, apparently, factors in the head which evoke from the competent mesenchyme just sufficient chondrification to provide a normal skeleton (see Hörstadius, 1950). None the less, it was thought worth while to perform transplantation experiments in which anterior or posterior cranial crest was grafted homoplastically to the branchial region, in the hope that local conditions at the graft site might occasionally prevent such regulating influences from operating and so permit the formation of supernumerary cartilages from any graft cells that might have cartilage-forming capacity.



TEXT-FIG. 5. Homoplastic transplantation of anterior cranial neural crest to the branchial region.

In the event 24 hosts bearing grafts of anterior cranial crest (see Text-fig. 5) and 26 bearing posterior cranial crest (see Text-fig. 6) lived long enough for study. The position of the grafts varied somewhat from individual to individual, some lying dorsally between the myotomes and the skin, others laterally in the pharynx wall, and still others ventrally near or beneath the endostyle. The graft sites were marked as the centres of outgrowths of melanophores for a short period, but it soon became impossible to differentiate between those of the host and those of graft origin. The graft itself, however, remained more deeply pigmented than the surrounding tissues.



TEXT-FIG. 6. Homoplastic transplantation of posterior cranial neural crest to the branchial region.

Histological examination of the hosts revealed that in no case was the host's visceral skeleton abnormally large or supplemented by additional fragments of cartilage. The grafts had, in fact, differentiated in precisely the same way as those placed in the trunk. One finding, confined to four animals, is worth stressing. In these cases the graft lay laterally in the pharynx wall and formed a massive

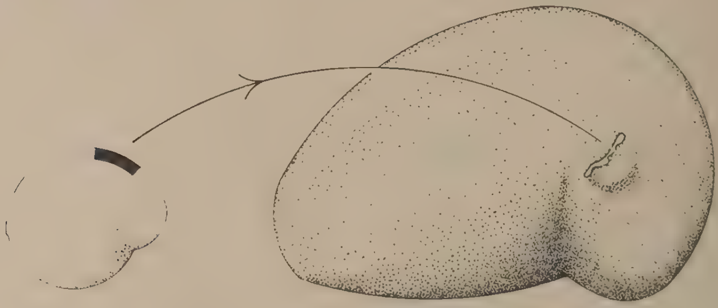
addition to the tissues normally separating branchial ectoderm from endoderm. In these animals the host branchial cartilage was missing or reduced in the arch in which the graft lay. This fact will be discussed below.

III. *Xenoplastic implantation of cranial neural crest into the head of urodele neurulae*

It is probable that in the case of homoplastic grafts of cranial neural crest to the head there is a considerable intermingling of ectomesenchyme cells from host and donor. The dual origin of the chimaeric structures so produced will not, however, be evident because of the difficulty of identifying donor cells by histological methods. This difficulty may not apply in xenoplastic combinations (cf. Raven, 1936). For this reason the capacity of lamprey neural crest to form cartilage was tested by implanting small pieces of it into the branchial region in newt neurulae. The hosts were allowed to live for at least 25 days after the operation by which time their own head skeletons were chondrified and the lamprey donors would have reached a length of 7 mm. had they been allowed to develop normally. The newt larvae were then killed and their heads sectioned.

(*a*) *Posterior cranial neural crest*

It was found that lamprey embryonic tissues would not heal into, or establish epithelial continuity with, those of newt neurulae at all satisfactorily, and the operation had therefore to consist of implanting the small piece of lamprey crest beneath the host's ectoderm as shown in Text-fig. 7. Even so, the proportion of extruded grafts was high, and only eighteen hosts with grafts survived until the



TEXT-FIG. 7. Xenoplastic implantation of anterior cranial neural crest from lamprey into the head of a urodele embryo.

stage required. In none of these animals was the graft evident while the host was alive, but this is not surprising in view of the relatively small amount of lamprey tissue within the head of the host.

In section the cells of the host were perfectly distinguishable from those of the donor. Not only were the lamprey cells very much smaller than those of their

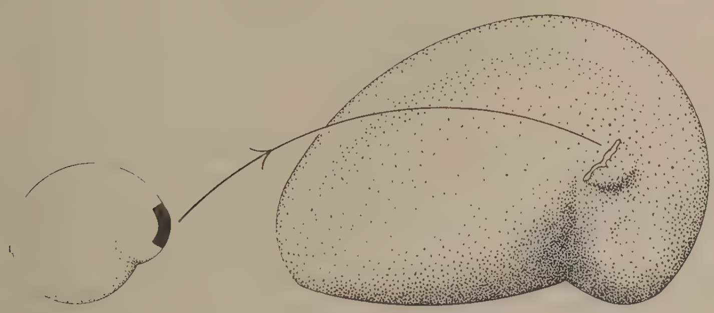
hosts, but they also took up the stain much less. In every case the donor cells appeared healthy, and mitotic figures were not uncommon among them. Nor, in most cases, did the host tissues in the neighbourhood of the graft show any reaction to its presence.

Most of the grafts had formed neural vesicles of remarkably normal appearance; surrounding these there was always a more or less dense population of lamprey cells either completely isolated from one another or in small groups. Where no neural formation was present the implant was represented only by such scattered cells. Only in two animals were melanophores of lamprey origin unmistakably present, and in both cases there were very few of them.

In 6 of the 18 hosts examined there were, however, small nodules of lamprey cartilage. They varied in size from one which, containing less than six cells, was smaller than the nucleus of a neighbouring newt cell to one roughly ten times as large. Two were in close contact with host cartilage, but the others lay free in the newt's mesenchyme. The appearance of three of them is shown in Plate 1, figs. A, B, C.

(*b*) *Anterior cranial neural crest*

The newts carrying implants of anterior cranial neural crest which lived long enough to be sectioned numbered, unfortunately, only nine. The operation (see



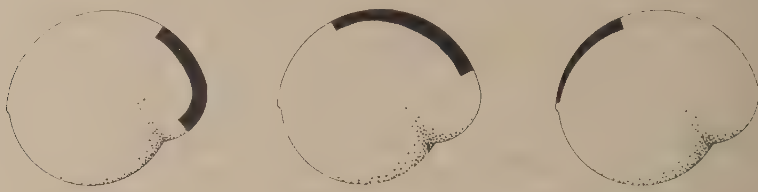
TEXT-FIG. 8. Xenoplastic implantation of posterior cranial neural crest from lamprey into the head of a urodele embryo.

Text-fig. 8), subsequent history, and general appearance of the grafts in section differed in no way from those of the series with posterior cranial crest implants. In none of the nine, however, was a single nodule of cartilage found. How far this result may be regarded as significant will be discussed below.

IV. *The extirpation of the neural crest in the head and in the trunk*

Loss of small lengths of the neural crest of the neurula may be made good by the backward and forward migration of cells from the two ends of the operated region. The masking of the effect of the operation in this way will, of course, be

less likely to occur the greater the amount of material moved. Experiments were therefore performed in which considerable lengths of crest were removed (see Text-figs. 9, 10, 11) and the animals cultured into larval life. They were killed and sectioned after reaching a length of 7 mm. or more.



TEXT-FIG. 9 TEXT-FIG. 10 TEXT-FIG. 11

TEXT-FIG. 9. Total bilateral removal of the anterior cranial neural crest.
TEXT-FIG. 10. Total bilateral removal of the posterior cranial neural crest.
TEXT-FIG. 11. Total bilateral removal of the trunk neural crest over much of its length.

(*a*) *Removal of anterior cranial neural crest*

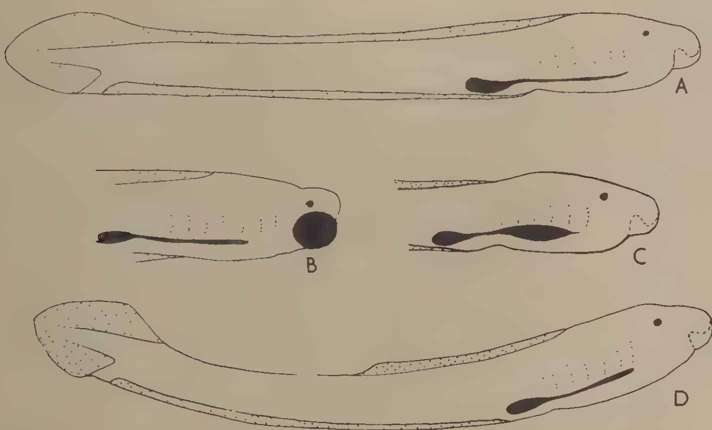
The animals that had suffered the operation shown in Text-fig. 9 showed, while alive, a varying degree of abnormality in the anterior head region. Some were almost normal in appearance while others had heads reduced at the anterior end, particularly dorsally, in the manner shown in Text-fig. 12B. Most typical was the congestion with blood of the lateral parts of the oral hood. The bright red tip to the head which this produced was clearly visible on macroscopic examination.

Twenty-one animals lived long enough to be worth sectioning. The sections revealed that the trabeculae cranii (anterior parachordals) were in every case present and normal in appearance from their posterior limit, abutting or fused with the parachordals, to as far forward as the anterior tip of the notochord or the level of the eyes. At this level they normally diverge laterally to a considerable extent and lie very close to the eye-cups themselves. As a result of the general foreshortening of the rostral region in this series, it was frequently found that the eyes were displaced backwards a little and lay in the same transverse plane as the tip of the notochord. Thus in some of the animals the trabeculae could be identified to a level somewhat anterior to the eyes. It is important to emphasize that although they could rarely be traced farther forward than this, the explanation is not necessarily that their embryonic anlagen were removed. At the age at which the animals were killed the trabeculae are well chondrified only to this level and farther forward they, and the trabecular commissure, are represented by a thin strand of cells whose certain identification in the very abnormal head of experimental animals is not possible.

In several of the animals there were found, either unilaterally or bilaterally, small nodules or plates of cartilage in the oral hood wall at the level of the eyes. These seemed in some cases to have a pro-cartilaginous connexion with the

trabeculae dorsally. Their morphological status is unclear, but they presumably owe their origin to the abnormal situation in the anterior head although they may well be the equivalents to the pieces of cartilage found in two of his wild-caught ammocoetes by Johnels (1948).

Of the other features in these animals worthy of notice, the most striking is the reduction of the oral hood-walls into small sacs of blood. The sections showed that much of this blood was extravasated, but it is, in part at least, surrounded by a thin endothelium and this suggests that the effect of the operation on the blood vascular system is an enormous dilation of the normally very fine vessels in this region. It must be emphasized that only the ventral part of the head was affected —dorsally the vessels are normal.



TEXT-FIG. 12. The effects of neural crest removal as seen in living larvae. In black are shown the main concentrations of blood visible on external examination. A, normal control larva. B, larva lacking anterior cranial neural crest. C, larva lacking posterior cranial neural crest. D, larva lacking neural crest in posterior trunk.

Other abnormalities concern the eyes, the epidermis, and the profundus and trigeminus ganglia. The eyes are quite normal in appearance in about half the series, in the others they are large, and in some they present a quite extraordinary vesicular appearance—the optic cup being, in effect, opened out to form a vesicle against the distal wall of which the lens rudiment is applied.

Of the ganglia of the dorsal root series the first, the profundus, was affected in nearly every case. Usually much reduced in size, it was almost absent in two individuals. The trigeminus was less often, and less seriously, affected. In the few cases where it was small the reason lies, presumably, in the unusual extent of the region of operation.

Finally it was observed that the epidermis of the ventral side of the ammocoete in the region of operation was frequently abnormal, and appeared, histo-

logically, reminiscent of the 'atypical epidermis' into which isolated gastrula ectoderm of amphibians develops. It would be wrong, however, to assume that this is directly due to the absence of underlying cells of neural crest origin, since a similar pathological appearance of ammocoete epidermis may occur in a number of different unfavourable conditions of culture.

These results are illustrated in Plate 2, figs. E, F.

(*b*) *Removal of posterior cranial neural crest*

Twenty-six animals which had suffered the removal of the neural crest over the whole of the branchial region, as shown in Text-fig. 10, were later studied histologically. Most, but not all, of them displayed while still alive a conspicuous red streak ventral to the gills (see Text-fig. 12c). This proved to be due to the dilation of the ventral aorta (or aortae), which, however, was (or were) invariably still confined in a continuous endothelium. The afferent branchial arteries were similarly affected, but neither the dorsal aorta nor the venous system showed any signs of abnormality. Close comparison of these dilated vessels with normal controls, failed to reveal any very obvious difference in the thickness of the walls.

The branchial basket cartilages, at this stage represented by vertical bars with hypo- and epi-trematic projections but not yet a complete basketwork, varied in condition from animal to animal. In some (six) animals they were completely lacking. In several more there were only small nodules of cartilage to be found, and in yet others it was complete anteriorly or posteriorly but absent in one or more visceral arches. In only one animal did it appear normal. This variation must be attributed to lack of precision in performing the operations. The parachordals were never affected.

The hind-brain and spinal cord were always normal in structure, but the ganglia of the dorsal root series were affected to a varying degree. The glossopharyngeus and vagus were occasionally smaller than normal, and the first five spinal ganglia were absent in some specimens. It is, however, remarkable that in some individuals the ventral symptoms of crest removal (absence of cartilage and dilated ventral aorta) were combined with the presence of spinal ganglia in the corresponding region. This suggests that the cells destined to form the ganglia lie ventral to those which will give rise to the ectomesenchyme in the neurula, and that it is therefore possible to remove the latter without the former.

The dorsal fin of the normal young ammocoete has its origin anteriorly at the level of the fourth gill opening. It thus overlaps the branchial region for a short part of its length. It was absent in this region in a number of the animals of this series (see Text-fig. 12c).

The results obtained from this series are illustrated in Plate 2, figs. G, H.

(*c*) *Removal of neural crest in the trunk region*

A small series of animals suffered the operation shown in Text-fig. 11. Sixteen of them survived long enough to be worth study. Of these a few were normal in

appearance, but most showed a more or less marked reduction in the size of the dorsal fin over some part of the trunk (see Text-fig. 12D). Such reduction never affected the fin behind the cloaca. There were no other visible departures from the normal in the external appearance of these animals.[1]

Sections showed that the operation had in some animals, and in part of the trunk region of them, been too drastic in that the whole of the spinal cord was missing. At the other extreme several animals were completely normal. Still others showed the spinal cord reduced in cross-sectional area over part of its length though nowhere interrupted. Finally a few showed a spinal cord of normal appearance, but with defects in other structures. In these alone, we must presume, the intention of the experiment had been fully realized and the neural crest had been extirpated, but the rest of the neural rod left substantially undisturbed.

The condition of these animals, and of the appropriate regions of others in which the operation had been too drastic in particular places only, is simply described. The dorsal fin with its contained mesenchyme is missing. The somites usually meet mid-dorsally over the spinal cord in a space normally occupied by connective tissue. The epidermis near the mid-dorsal line is sometimes, but not always, thickened and abnormal in appearance. The dorsal root ganglia are missing.

The spinal cord itself may appear normal in cross-section, and may lie, as is normal, within a canal sparsely populated with cells and surrounded by a connective tissue sheath. However in some cases, and always where the spinal cord is itself much reduced in diameter, this canal has been lost, the fairly conspicuous connective tissue sheath has been replaced by a very fine epithelium, and the spinal cord is separated by this alone from the somites and chorda which crowd around it.

These results are illustrated in Plate 2, fig. I.

DISCUSSION

Before attempting a general comparison between the properties of the neural crest in lampreys and other vertebrates it is necessary to assess the contributions made by the crest to structures and tissues in the ammocoete in the light of the experimental results described.

1. *The branchial basket*

Strictly speaking this work does not relate to the whole branchial basket, since this structure is not, in normal development, completed until the ammocoete is

[1] It might be expected that the development of the melanophores of the trunk would be affected. Unfortunately the animals of this series, together with their controls, were unusually retarded in the development of their pigmentation. At death the controls were only just beginning to show melanophores in the trunk, and the absence of pigmentation in the experimental series cannot therefore be regarded as significant.

somewhat older than the oldest of the experimental animals. Thus the young ammocoete of 7 mm. total length has well-defined cartilaginous elements representing the vertical bars, the ventral bar, and the subchordal bar. The epitrematic and hypotrematic bars are, however, not yet complete. Consequently the results of these experiments must be taken as referring primarily to the first formed parts of the basket, and in particular, to the vertical bars which are already chondrifying when the animal is 6 mm. in length. It may be added that there is little reason to believe that the branchial basket proper has a dual embryonic origin.

It is clear that the simplest interpretation of the experimental results is that the branchial basket is of neural crest origin, and hence that the provisional conclusion drawn from the earlier experiments (Newth, 1951) was wrong. All the results are consistent with this view, only excepting the single aberrant animal in the series of grafts of pharynx wall without neural crest to the flank. The explanation of this result offered above, namely that by operative error some crest cells were included in the graft, is almost certainly correct.

It is only fair to point out, however, that an alternative explanation is possible. This is that the crest induces the formation of the branchial basket (thus explaining the results of the defect experiments), that it forms cartilage in the urodele head because of inductive influences there present but absent in lampreys, and that the grafts of pharynx wall less crest did not form cartilage because of the absence of the inducing stimulus provided by cranial crest cells. On balance this explanation has little to commend it in comparison with the other.

We may now consider the four cases in which the formation of visceral arch skeleton was inhibited by the presence in the arch of massive grafts of cranial crest. The explanation of this, seemingly odd, result is perhaps to be found in some experiments performed by Damas (1951) on the effect of light on young lamprey embryos. He describes three animals which, having been exposed to light while very young, developed marked abnormalities in the head, including deficiencies of their visceral skeleton. These he interprets as due to a failure to establish contact between branchial ectoderm and endoderm during gill pouch formation. If he is right in thinking that such contact has an inductive significance and that in its absence no visceral cartilage will form, then it is not surprising that the presence of a large neural formation in the visceral arch should block, mechanically, this necessary inductive contact.

We may summarize by saying that the experiments here reported confirm the view held by Koltzoff and Damas that the branchial basket is of crest origin, that they show that in lampreys, as in Amphibia, the pre-migratory crest is not determined to form cartilage although it is competent to do so, that they suggest that the influences in the head that promote chondrification of the visceral skeleton behave as do those of Amphibia in that quantitative regulation occurs in the presence of an excess of competent crest cells, and finally that they give some support to Damas's conception of the importance of contact between endoderm

and ectoderm in the branchial region for the normal development of the visceral skeleton.

2. *The trabeculae cranii (anterior parachordals)*

These structures are, in the young ammocoete of 6–8 mm. in length, two cartilaginous bars lying posteriorly against the anterior end of the notochord and establishing contact with the parachordals proper with which they later fuse completely. As the animal grows they extend anteriorly in front of the notochord diverging laterally at the level of the eyes but finally converging to join medially beneath the fore-brain by a trabecular commissure.

The difficulty of assessing the value of the experiments designed to test their claim to be neural crest derivatives is that here we have to take seriously the possibility of their having a dual origin. Not only is this the case in Amphibia, but Johnels (1948), who believes that they are probably derived from paraxial mesoderm posteriorly, is prepared to admit that their anterior extension and the formation of the commissure may be at the expense of ectomesenchyme which is abundant in this region.

Now the experiments here reported can only be held to apply with certainty to the trabeculae from their point of origin posteriorly as far forward as the eyes, since only this part of them is well chondrified and easily identified at the age at which the animals were killed. It is true that in normal controls of 7–8 mm. in total length it is quite possible to make out the slender pro-cartilaginous strand that represents the future commissure and extreme anterior end of the trabeculae, but in the experimental animals that are most relevant to this problem, those of the extirpation series, the general deformations of the anterior head are sufficiently great to make definite assertions about the presence of this anterior region unsafe.

What then do the experiments show? It seems impossible to deny that the extirpation experiments point strongly to the independence of the posterior part of the trabeculae cranii of the neural crest. In animals that show the most marked reactions to the operation in other ways, e.g. by the presence of huge pools of blood in the wall of the oral hood, the trabeculae are not visibly affected. The xenoplastic transplantation experiments add force to this view. As they stand, if we take the proportion of xenoplastic grafts of posterior cranial crest which gave a true answer as our base, they place the probability of the negative result's being misleading at 0·063. It should be added that even if a very small proportion of these experiments had yielded a positive, i.e. that cartilage had been formed in the newt head from anterior cranial crest, it would still be possible that this was due to operative error in including some of the presumptive branchial basket crest in the implant. Only if the implants from anterior head crest had provided cartilage at least nearly as often as the posterior crest would the result merit counting as a positive.

In consequence we must assume, in agreement with Koltzoff and Johnels, that

the so-called trabeculae cranii of lampreys are not of neural crest origin throughout their whole length. Are they derived from ectomesenchyme from a different source? This remains possible though Koltzoff thought them to come from cells of the sclerotome of the mandibular segment and with this finding Johnels is inclined to agree.

3. *Dorsal root ganglia*

Both the spinal ganglia and the dorsal root ganglia of the head are affected by extirpation of the neural crest. In the case of the spinal ganglia the demonstration of their crest origin must depend upon those cases in which the spinal cord in the region of operation is complete, but the ganglia are absent. It was inevitable that in removing so small and tenuous a tissue as the pre-migratory crest in the trunk region there should be many cases of incomplete removal and others in which the bulk of the spinal cord was removed with the crest. What is crucial is that there are cases in which the cord is complete and in which the ganglia are missing. Had the ganglia been derived from cells migrating out from the cord itself this result would not be at all likely.

The position in the head remains far from clear. In the experiments reported in Newth (1951) all of the dorsal root series in the head (V_1, V_2, VII, IX, and X) were in one or more animals affected by crest extirpation. In the most extreme case the vagus ganglion on one side of an experimental animal was completely missing. Now it is generally probable, and Damas's observations confirm this, that there is a more or less extensive placodal contribution to all the dorsal root ganglia of the head. We could reconcile this with the experimental results by assuming that the crest induces the placodal contribution, or is in some other way necessary for its organization into a ganglionic mass. But in this case we have to explain the presence of ganglia of reduced size after total crest extirpation. It seems more likely that a too radical surgery has, in some cases, removed the placode as well as the crest.

4. *Melanophores*

The present results do not add anything to the earlier ones. All homoplastic grafts of neural crest clearly provided melanophores to the surrounding tissues. Whether or not other parts of the central nervous system can provide melanoblasts (as in the experiments of Lopashov, 1944, on teleosts and Niu, 1947, on amphibians) is not known. If they can it would be particularly interesting to know whether they do in normal development.

5. *The blood vascular system*

The discovery that crest extirpation in the head resulted in a marked dilation of the ventral arteries in the region of the defect was unexpected. This does not, so far as I know, occur in amphibia, nor did it occur in the trunk region of lampreys. There are two simple, and not mutually exclusive, explanations. The

first is that in the absence of the normal density of neural crest mesenchyme blood-vessels dilate under the functional stress produced by the pressure of the blood within them although their walls are of normal structure. The second is that the walls of the vessels affected are, in part at least, normally of crest origin and are so weakened by its absence that they dilate where the pressure is high.

Damas (1944) mentioned that the sheath of the ventral aorta was formed from ectomesenchyme. I take these experiments to be a confirmation of his view, and the normal appearance of the walls of the ventral aorta in my experimental animals to be the result of secondary regulative processes late in embryonic or early in larval life.

6. *The dorsal fin mesenchyme*

The effect of removing the trunk neural crest upon the dorsal fin is completely in accord with amphibian experience. We may therefore provisionally conclude that the fin-mesenchyme is of crest origin and that it also plays a part (cf. Bodenstein, 1952, for an analysis of the amphibian situation) in the induction of the fin. Further experiments would, however, be needed to establish this beyond doubt.

It is thus clear that the neural crest of the lamprey embryo shares important properties with that of the amphibians and, for that matter, with that of all vertebrates in which it has been studied. Thanks above all to Conklin, Vogt, and Pasteels we are in possession of a clear understanding of the fundamental unity of the morphogenetic movements mediating gastrulation and neurulation in chordate animals. For the craniate vertebrates, but not for pro-chordates, a further and subsequent displacement must be accepted as fundamental—the migration of the cells of the neural crest to colonize virtually every region of the embryo between the classical 'germ-layers', there to differentiate into a variety of non-neural tissues.

But if we assume, as we fairly may, that the neural crest of the common ancestors of lampreys and gnathostomes was at all similar to the crest of today, then it means that this distinction between craniate and pro-chordate embryos was already firmly established in the times when Osteostracii flourished, and possibly earlier still. It then becomes difficult to accept the argument of Garstang (made public and supported by Hardy, 1954) for regarding Amphioxus as the descendant of the neotenic ammocoete of an osteostracan. The last thing that such an ammocoete would lose by neoteny would be an embryonic tissue with such diverse and important derivatives as the neural crest.

But if, so far, the lamprey embryo fails to provide evidence of intermediate stages in the evolutionary development of the neural crest, can it help to determine the morphological status of the skeletal elements of the head to which it does, or does not, give rise? Had the branchial basket proved to be of non-neural crest origin this fact would, I think, have supported, though not conclusively, those who have held that this structure is not the homologue of the splanchno-

cranium of gnathostomes. That it is of crest origin, on the other hand, does not give equivalent force to the opposing view, since it is far commoner for non-homologous skeletal elements to share an embryonic origin (e.g. the paraxial mesoderm) than for homologous ones to have different embryonic origins.

The relevance of the embryological origin of the so-called trabeculae cranii of lampreys to their morphological status has been discussed in Newth (1951). Unfortunately the decisive information, on the origin of their most anterior parts, is still lacking. If the crest should, on further investigation, prove to contribute to them anteriorly then it will clearly be rather difficult to doubt their homology with the trabeculae of gnathostomes. If, on the other hand, it contributes nothing we may well, following Sewertzoff and de Beer, hold them to be structures *sui generis*.

SUMMARY

1. Those parts of the branchial basket of the ammocoete larva that have chondrified by the time the animal is 7 mm. long are of neural crest origin.

2. Those parts of the trabeculae cranii (anterior parachordals) of the ammocoete that have chondrified by this time are not of neural crest origin, nor do they depend upon the presence of the crest for their normal formation.

3. The factors governing the development of the visceral arch skeleton are, as far as is known, equivalent to those operating in Amphibia. The pre-migratory crest of the branchial region is competent to form cartilage, but not determined to do so.

4. It is probable that the normal development of the visceral arch skeleton is dependent upon the early establishment of contact between branchial ectoderm and endoderm.

5. The cranial neural crest is necessary for the normal development of the ventral arteries of the head. In its absence they are much dilated. It is probable that the walls of these vessels are normally, at least in part, of crest origin.

6. In the absence of the neural crest in the trunk region the dorsal fin of the larva is not formed.

7. Absence of the neural crest leads to absence of the dorsal root ganglia in the trunk and, usually, to a reduction in size of those of the head.

8. The neural crest is a source, if not the only source, of larval melanophores.

Among many colleagues who have helped me by providing living material I am especially indebted to Dr. R. A. Kille, Dr. H. Spurway, and Professor D. M. Steven.

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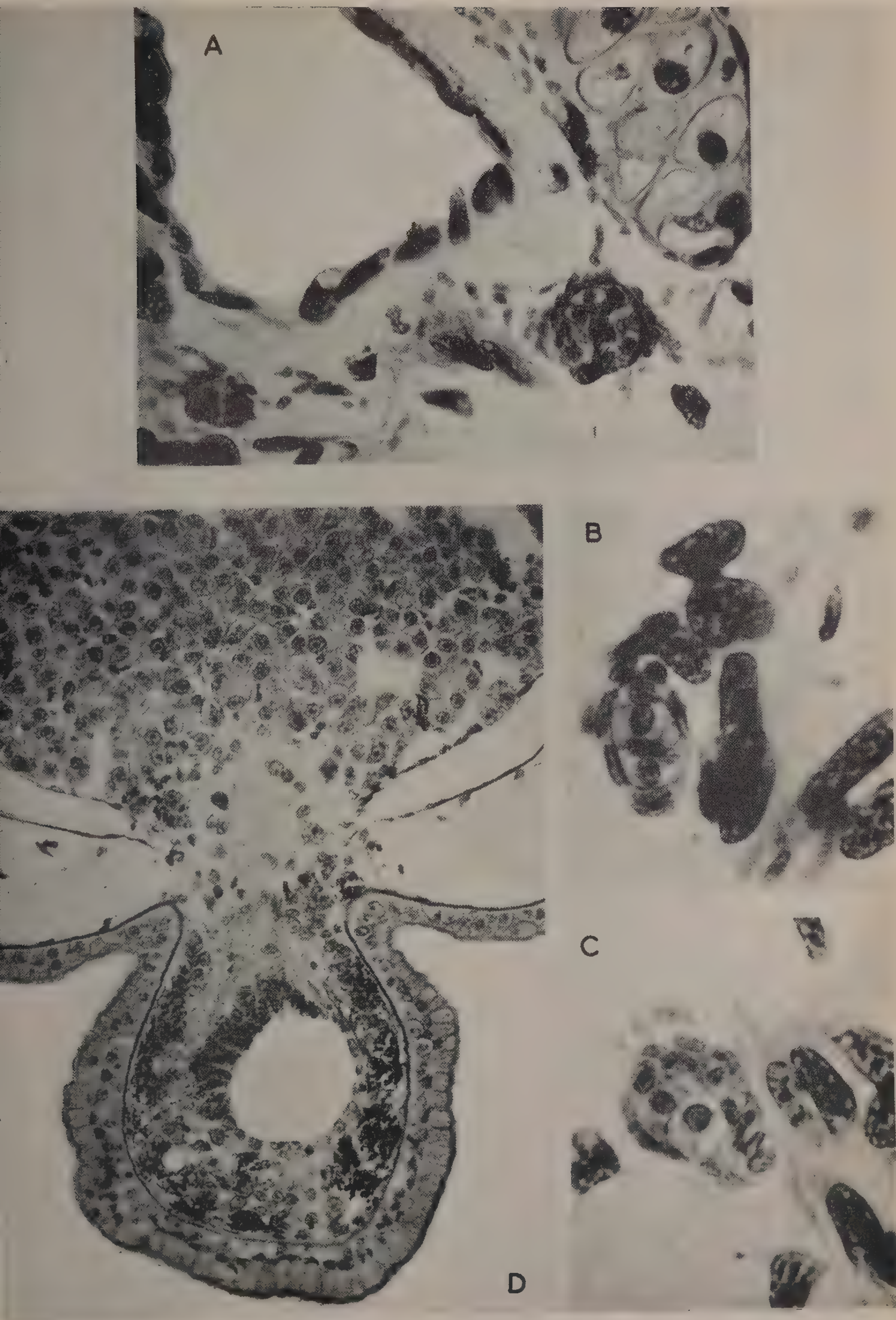
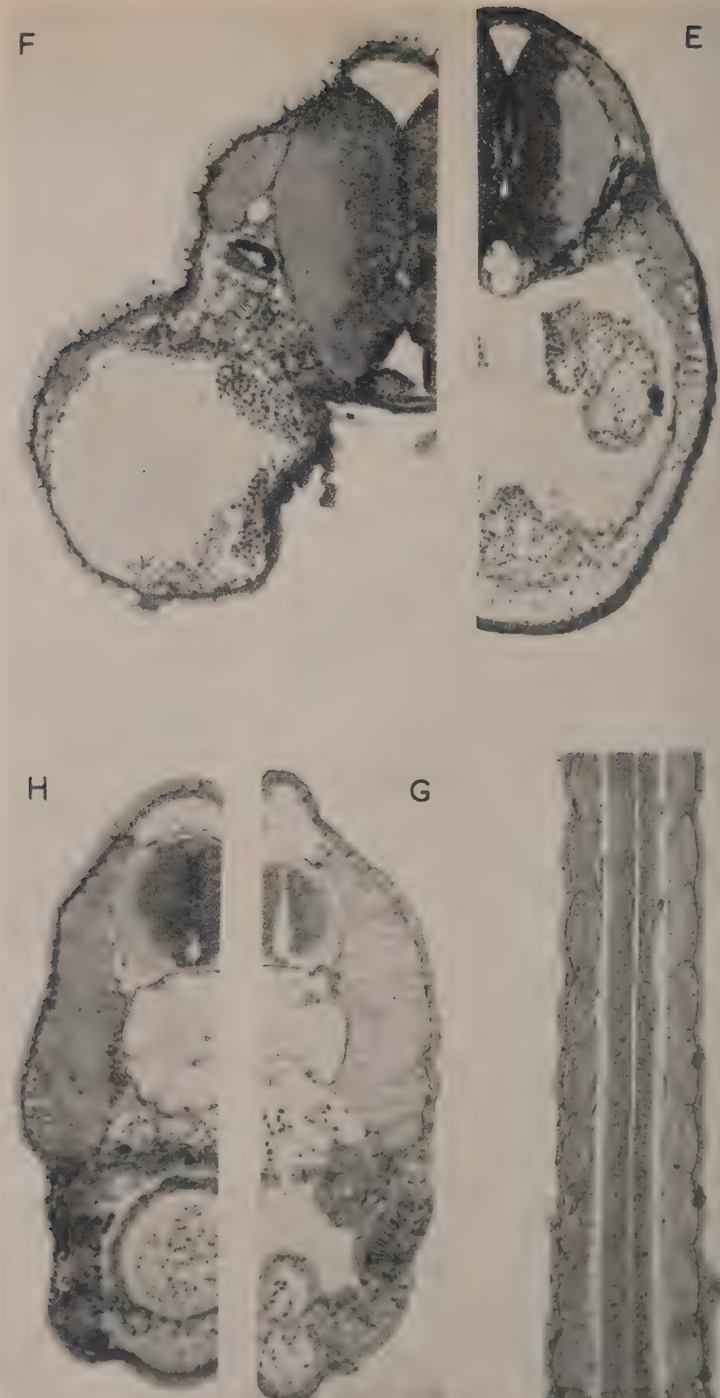


D. R. NEWTH

Plate 1



D. R. NEWTH

Plate 2

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EXPLANATION OF PLATES

PLATE 1

FIGS. A, B, C. Three cases, from different hosts, of the formation of cartilage by cells of the posterior cranial neural crest of lamprey embryos implanted into the head of newts. The lamprey cell nuclei are very much smaller than those of the newt hosts.

FIG. D. The result of homoplastic transplantation of posterior cranial neural crest of lamprey into the flank. The graft has given rise to neural tissue, mesenchyme and melanophores, but not to cartilage.

PLATE 2

FIGS. E, F. Comparison between the anterior head of a control ammocoete (E) and one which lacked anterior cranial neural crest (F).

FIGS. G, H. Comparison between the branchial region of a control ammocoete (G) and one lacking posterior cranial neural crest (H).

FIG. I. The consequences of removal of neural crest in the trunk region. Note absence of dorsal root ganglia in the region of operation.

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The Distribution of Placental Scars of First and Second Pregnancies in the Rat

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WITH TWO PLATES

INTRODUCTION

THE purpose of this study is to investigate possible interrelationships between successive sets of placental scars in the laboratory white rat (*Rattus norvegicus*). The primary problem has been to determine if placental scars formed as a result of a second pregnancy are ever superposed upon those of a first pregnancy. An attempt has also been made to learn if there are any changes in location or spacing of second sets of scars when they are compared with the distribution of scars remaining from a previous pregnancy.

A placental scar may be defined as a pigmented area of the uterus occurring at the site of a previous placenta. In the rat the placental scars appear as orange to dark brown pigmented spots along the mesometrial border of the uterus. The scars are produced by an accumulation of hemosiderin in the cells of the reticulo-endothelial system. These cells are concentrated in the area between the longitudinal and circular muscle layers as well as in the deeper part of the endometrium. As the age of the scars increases the pigmented areas decrease in size and become darker in colour. It is thus relatively easy to distinguish old placental scars from very new ones. Since the rat placenta forms at the same uterine level and opposite the antimesometrial implantation site, inferences about the spacing of blastocysts at implantation can be drawn from studies of the spacing of scars.

METHODS

Virgin rats approximately three months in age and of the Wistar strain were used in this study. These animals were bred at known dates and isolated after breeding. The number of young born in each litter and the date of birth were recorded. Litters were weaned on day 21 and at this time the first laparotomy was performed on the uniparous females. The reproductive tract was exposed and examined by holding the mesometrium and uterine horn between the fingers

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in front of a strong light. Placental scars appeared as dark spots in sharp contrast to the pink uterine horn. The number and approximate location of scars in each horn were recorded.

The uniparous female rats were bred a second time 10 to 20 days after the first laparotomy. On day 12 of this pregnancy, a second laparotomy was performed and the number of gestation sacs present was determined. Placental scars of the first pregnancy were not clearly visible at this time. The animals were permitted to go to term and then were sacrificed on post-partum day 12.

The uteri were removed, pinned in a dissecting pan by placing a pin at each end of the horn, and flooded with 10 per cent. formalin. They were later bleached with hydrogen peroxide to increase the contrast of the placental scars with the surrounding tissue. Measurements of the total length of each uterine horn and the intervals between scars were made by calipers. The diameters of placental scars were measured by using a dissection microscope and ocular micrometer. The measurements of scar lengths and intervals between scars in each horn were converted to percentages of the total length of that horn, so that comparisons between horns could be made.

Scars of the first and second pregnancy were usually easily differentiated. Those of the first pregnancy (old scars) were approximately one half as large as the new scars, dark brown to black in colour, and restricted to the mesometrio-uterine junction. The new scars were large, swollen areas extending well into the mesometrium and of yellow or golden colour.

A total of 51 experimental animals was used, but results are presented for only 61 horns. Other horns were excluded because of failure to become pregnant, infection, post-operative adhesion, or similarity of appearance of old and new placental scars.

The technique of pinning and stretching the uterine horns may be subject to criticism because conclusions drawn from the measurements would be invalidated if the elongation were not uniform in all segments of the horn. The method was checked by applying spots of dye near both cervical and oviducal ends of the horns of two females and measuring *in situ* the length of the horns, the interval between spots, and the distance from each spot to the nearest end of the uterus. The measurements were repeated after the tracts were removed, stretched, pinned, and fixed. When the measurements were converted to percentages, it was found that the maximum variation in intervals was only 1·9 per cent. It is concluded that stretching was essentially uniform throughout the tract and that the method is sufficiently accurate to warrant comparisons of measurements.

RESULTS

Superposition and merging of scars

The number of placental scars observed in the preserved uteri varied from the number expected. During the first laparotomy 312 old scars were recorded, while 368 developing gestation sacs were noted during the second laparotomy. At the

final examination only 280 old placental scars were found (thus 32 or 10 per cent. were missing). One or more placental scars were missing in 17 of the 61 uterine horns.

In one horn, the number of old placental scars was greater than expected. Only three old placental scars were seen during the first laparotomy, but four old placental scars were found in the preserved tract. The most probable explanation is that one placental scar had been overlooked during the first laparotomy. The extra scar was located near the cervix, which is the most difficult area to see at laparotomy.

The number of new placental scars found was 369, as compared to 368 expected. The discrepancy occurred in a horn where the additional scar was located at the cervical end and almost within the cervix. Again it is assumed that the embryo count was incorrect.

Gross examination and comparison of the number and position of visible placental scars with the diagrams showing scar distribution at the first laparotomy made it possible to predict probable locations for 'missing' scars. A close examination of the suspected scars often revealed that overlapping was incomplete and the double scars were larger than single new ones (Plate 1). Frequently a dark streak of pigment was also apparent at the junction of the swollen new scar and the mesometrium. Often the position of the missing scars was doubtful even though the number which should have been present was known. Identification of double scars was made more difficult because some single old or new placental scars had two areas of pigment concentration, making them appear double (Plate 1). This pattern was produced when pigment accumulated inside the circular muscle stratum in the deeper stroma of the mesometrial side and also in the area between the circular and longitudinal muscle layers. No consistent diagnostic character of the double scar was found in the gross studies. Unless the previous reproductive history of an animal were precisely known, it would be impossible to determine from gross examination of placental scars the actual number of implantation sites represented.

Histological preparation of examples of old, new, and superposed scars were made. These scars were serially sectioned and stained with haematoxylin and eosin. Microscopic study showed that superposed sites could be clearly distinguished (Plate 2, figs. A–F). Pigment accumulates as large granules in macrophages lying between the circular and longitudinal muscle layers (Plate 2, fig. D) in old scars. In new scars, these cells are larger, the cytoplasm more abundant, and the pigment granules much smaller (Plate 2, fig. E). A more detailed description of the histology of placental scars is given by Conaway (1955). Both types of pigmented cells are apparent in superposed scars (Plate 2, fig. F). The small cells laden with large granules occupy a more peripheral position and lie just under the longitudinal muscle. This distribution produces the visible dark line observed in gross examination. The large cells with small granules are more abundant and more centrally located. Superposed scars can thus be clearly recognized under

the conditions of this experiment. If both scars were much regressed, however, it is doubtful that microscopic examination would differentiate between them.

The expected frequency of merging first and second pregnancy scars

It has been indicated that 32 of the 312 old scars were not visible as separate spots along the uterus. These concealed old scars were either in contact with or partially or completely overlapped by new scars.

A crude estimate of the expected number of old scars which would be so concealed can be made if it is assumed that the new embryos implant randomly.

TABLE 1

Frequency of merging new and old scars

No. old scars in horn	Percentage of horn length on which merger occurs	Total no. new scars	Expected no. merging new scars	Observed no. merging new scars
1	4·5	6	0·27	0
2	9·0	16	1·44	0
3	13·5	46	6·21	2
4	18·0	61	10·98	3
5	22·5	95	21·38	4
6	27·0	53	14·31	5
7	31·5	33	10·40	9
8	36·0	38	13·68	5
9	40·5	8	3·24	0
10	45·0	8	3·60	3
11	49·5	5	2·48	1
Totals	—	369	87·99	32

Merging of old and new scars will occur if the centre of the new scar is within a radius of itself from either edge of an old scar. The average diameter of old scars is 1·7 per cent. of the length of the horn and the average radius of new scars is 1·4 per cent. Therefore, merging of old and new scars will occur if the new scar lies within a segment equal to 4·5 per cent. of the uterine length. Each additional old scar adds 4·5 per cent. to this total length. The number of merging scar pairs expected for each group of uteri with the same number of old scars equals $N \times 0{\cdot}045 \times T$, where $N =$ number of old scars per horn, $0{\cdot}045 =$ the segment over which merger will occur for one old scar, and $T =$ total number of new scars in all horns with N old scars. As an example, the number of merging scar pairs in uteri with four old scars equals $4 \times 0{\cdot}045 \times 61 = 10{\cdot}98$. Therefore approximately 11 of the 61 new scars would be expected to merge with an equal number of old scars.

The above calculations were repeated for each old scar number class (Table 1). These calculations show that 87·99 merging pairs would be expected. This is considerably in excess of the 32 merging pairs observed. Table 1 also shows that

the number of superposed new scars observed is much less than expected when the number of old scars in the uterine horn is low. When five or more old scars are present the observed and expected frequency of merging are in somewhat closer agreement.

Distributional pattern of scars of first and second pregnancies

Comparisons of the intervals between old and new placental scars were made to determine if there were differences in the intervals between scars of the two pregnancies. The data were analysed by calculating variances of the intervals between scars for all sets of 3–8 old and 3–8 new scars (Table 2). The variances

TABLE 2

Intervals between placental scars

No. scars in set	*No. old scar intervals*	*No. new scar intervals*	s^2 *old intervals*	s^2 *new intervals*	'F' *new/old*
3	18	10	33·53	90·00	2·69*
4	30	18	27·41	71·35	2·61*
5	56	32	20·30	31·80	1·57
6	30	45	22·04	15·54	0·70
7	24	72	6·96	7·27	1·05
8	42	77	8·15	13·33	1·63*

* = 95 per cent. confidence level.

were greater in all sets of new scar intervals than the variances for the corresponding old scar intervals, except in the six-scar class where the reverse relationship occurred. The 'F' test indicates the difference was significant only in the three-, four-, and eight-scar classes, and approached significance in the five-scar class. This analysis indicates that there is probably greater variation in the intervals between scars in sets of three, four, five, and eight new scars than in similar sets of old scars.

There is a tendency for placental scars of both sets to occur much closer to the cervix than to the oviducts. A greater distance free from old placental scars occurred at the oviducal end in 87 per cent. of the 61 horns and the same distributional pattern for new scars was found in 73 per cent. of these horns. The distances from the first scar to the oviduct and the last scar to the cervix were divided into scar number classes for both old and new scars. Comparison of the means of these distances showed that with one exception the greatest scar-free distance is at the oviducal end in all twelve categories. The 't' test showed that six of these differences were highly significant (Table 3).

The data (Table 3) also suggest changes in the relationships of the terminal scar-free segments in second pregnancies as compared to first pregnancies. The terminal old scars tend to lie closer to the cervix but farther from the oviduct than corresponding new scars. When the means are compared, the only exception to

this trend is that the mean of the first pregnancy cervical segment is greater than the corresponding second pregnancy segment in the three-scar class. The 't' test shows that only the difference between the old and new oviducal segment in the

TABLE 3

Comparison of oviducal and cervical scar-free segment lengths

No. scars per horn	*No. horns old scars*	*No. horns new scars*	$\bar{X}$ *ovi. new*	$\bar{X}$ *ovi. old*	$\bar{X}$ *cer. new*	$\bar{X}$ *cer. old*	*'t' new ovi. new cer.*	*'t' old ovi. old cer.*	*'t' old ovi. new ovi.*	*'t' old cer. new cer.*
3	9	5	27·6±9·09	31·7±8·80	24·6±12·01	27·7±16·00	0·45	0·66	0·83	0·36
4	10	6	15·2±2·86	25·3±5·83	25·2±18·32	17·5±6·11	−0·42	2·97†	4·21†	−1·24
5	11	7	21·0±8·38	23·0±4·62	11·3±5·26	9·8±9·02	2·13	7·15†	0·69	−0·68
6	6	9	19·6±8·09	20·3±6·59	11·2±6·76	5·9±2·32	2·95*	5·05†	0·18	−1·82
7	4	12	15·1±4·36	17·3±3·96	7·8±3·58	5·4±1·74	4·50*	5·51†	0·89	−1·29
8	6	11	15·4±5·59	16·8±3·38	7·0±3·92	6·8±3·66	4·09*	4·95†	0·56	−0·10

cer. = cervical scar-free segment. *ovi.* = oviducal scar-free segment.

* = 95 per cent. confidence level. † = 99 per cent. confidence level.

four-scar class is significant. The uniform trend in the differences between the means, however, suggests that there may be a real difference in the pattern of distribution of the scars in the two pregnancies.

DISCUSSION

Some relationships of placental scars or implantation sites of later pregnancies to scars of previous pregnancies have been noted by other workers. Fortuyn

(1920) reported that in the mouse there was a tendency for the gestation sacs of a second pregnancy to occur mid-way between the 'brown cells' of the first pregnancy. In a later study, Fortuyn (1929) reported that when embryos of the striped hamster (*Cricetulus griseus*) implanted in areas other than mid-way between old placental scars, resorption always occurred. De Lange (1934) states that in the gundi (*Ctenodactylus gundi*) either implantation does not occur or an abnormal gestation sac is produced as long as placental scars are distinct in the uterus. He indicates, however, that if only a few gestation sacs are present placentae of the second pregnancy occur between or beside old placental scars.

Bull (1949) emphasized that implantation in rodents is antimesometrial and placentation mesometrial. Thus, even when pregnancies occur in rapid sequence the two endometrial regions concerned are well separated until the embryos of the second pregnancy establish placentae. This arrangement, together with rapid and complete regeneration of the endometrium at the old placental sites, should make it possible for embryos of successive litters to occupy the same sites. No evidence that this does occur was presented by Bull. Davis & Emlen (1948) suggested superposition of placental scars might occur in the rat, since they found multiparous rats with fewer placental scars than the recorded total of young born.

The data obtained in the present study show that placental scars of the second pregnancy were partially or completely superposed upon 10 per cent. of the first pregnancy scars. It was estimated that if the new placental scars were distributed at random 28 per cent. of the old scars (88 of the 312 total) would contact or be overlapped by the new scars. This estimate is obviously crude since clearly the embryos of a set do not implant at random but tend to space along the horn. There is also a general anterior shift in the position of the second pregnancy scars which would tend to reduce the amount of overlap. However, the estimate does suggest that there is no preferential implantation at the level of old sites and it is possible that these sites are less favourable for implantation.

The major changes in the distributional pattern of second pregnancy scars as compared to old scars are: (1) the intervals between new scars are more variable; (2) the last new scar tends to be farther from the cervix than the last old scar; (3) the first new scar tends to be closer to the oviduct than the corresponding old scar; (4) although the oviducal scar-free segment is longer than the cervical segment in both pregnancies, the difference is less in the second pregnancy. Thus, the second set of scars tends to be more centrally spaced along the length of the uterine horn.

Frazer (1955) has shown that rat embryos are not distributed equally along the uterine cornua. He found that more embryos were implanted in the caudal half than in the cranial half. These findings are in agreement with those presented here. He also noted that the difference was more apparent when the embryo number was low than when it was five or more. The present analysis shows that the difference between cranial and caudal scar-free segment lengths is actually

more marked when the scar number is high. The different methods of analysis used may account for this variation in findings, especially since Frazer apparently did not consider the number of pregnancies per animal as a factor.

The cause of the changes in distribution is not known. Possibly one factor may be the vascular pattern of the uterus. Various workers have suggested that implantation tends to occur in regions of greater vascularity or of local hyperemia (Mossman, 1937; Reynolds, 1949; and Young, 1952). Preliminary work suggests that in the virgin uterus the cervical portion of the uterine horn is more highly vascular than the oviducal region. Such a pattern fits the distribution of first pregnancy scars. If this vascular gradient becomes less marked as a result of pregnancy, or is destroyed, it would be expected that implantation would normally occur over more of the uterine length in the second pregnancy.

Data obtained in the present study fail to show whether or not normal embryo development occurs at the superposed sites of second pregnancies. Since placental scars are formed when a portion of the embryos of a litter undergo resorption any time after the seventh day of pregnancy (Conaway, 1955), the presence of a scar does not indicate the fate of the embryo. The number of young born was always less than the number of gestation sacs recorded at the second laparotomy in all animals where superposition was recorded. However, the number of young recorded for the animals in which superposition did not occur was also usually less than the number of gestation sacs noted. Probably embryo resorption was high in all of the experimental animals, as a result of the laparotomies. Also the number of young recorded is not an accurate measure of the young born since the counts were often made as late as twelve hours after birth.

SUMMARY

1. In albino rats placental scars of a second pregnancy partially or completely overlapped 32 of 312 old placental scars from a previous pregnancy in 61 uterine horns.

2. Gross recognition of the superposed scars was often impossible. Microscopic recognition of the superposed sites was possible under the conditions of the experiment.

3. The major changes in the distribution pattern of second pregnancy scars as compared to those of the first pregnancy are (*a*) the intervals between second pregnancy scars are more variable; (*b*) the most posterior new scar tends to be further from the cervix than the last first pregnancy scar; (*c*) the most anterior new scar tends to be closer to the oviduct than the corresponding first pregnancy scar; and (*d*) although the oviducal scar-free segment is longer than the cervical scar-free segment in both pregnancies, the difference is less in the second pregnancy.

We are indebted to Dr. H. W. Mossman for suggestions and comments. Dr.

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EXPLANATION OF PLATES

PLATE 1

Uteri showing placental scars. Specimens are cleared and photographed with transmitted light. Wratten 39 filter.

PLATE 2

FIG. A. Cross-section of uterus 4L through an unscarred area. The myometrium is unmodified and no pigment is seen. ×125.

FIG. B. Cross-section of uterus 12R through an area showing a superposed old and new scar. The region of the old scar (O) appears more dense than the new scar area (N). ×125.

FIG. C. Cross-section of uterus 4L through an unscarred area at the mesometrio-uterine junction. ×555.

FIG. D. Cross-section of uterus 4L through an old placental scar at the mesometrio-uterine junction. The large pigment granules are apparent. ×555.

FIG. E. Cross-section of uterus 4L through a new scar at the mesometrio-uterine junction. The hypertrophied cells are laden with fine pigment granules. ×555.

FIG. F. Cross-section of uterus 4L through superposed new scar at the mesometrio-uterine junction. The smaller cells and large pigment granules of the old scar are at the right while the large cells and small granules of the new scar are at the left of the field. ×555.

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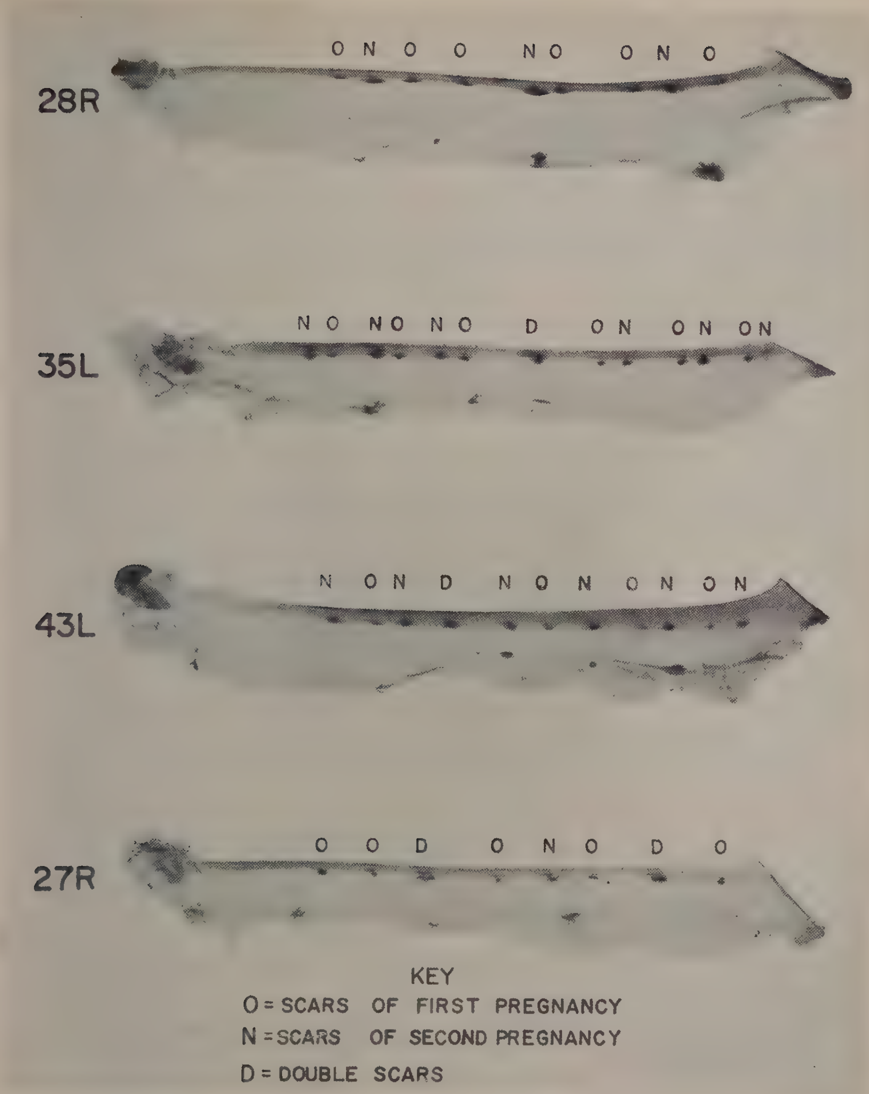

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Plate 1

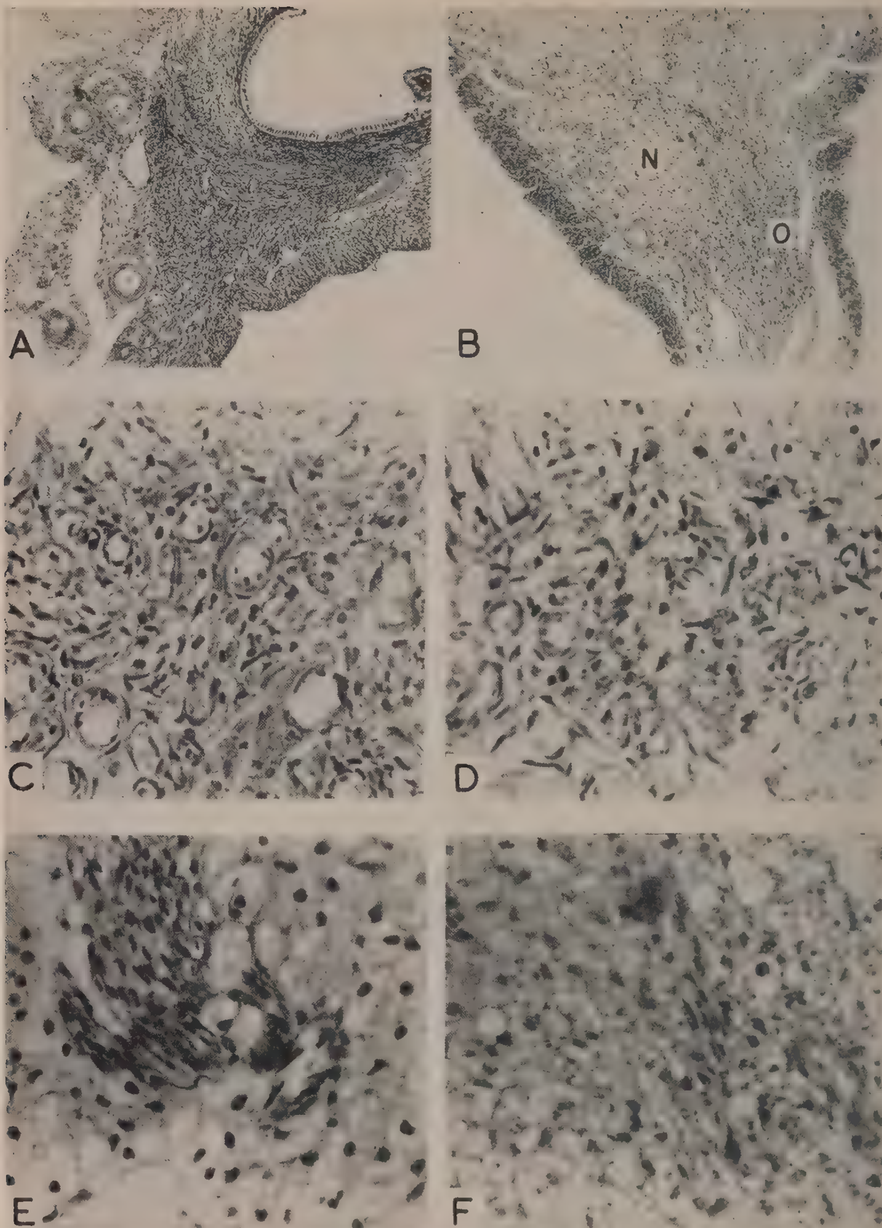

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Plate 2

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